

AD _____

Award Number: W81XWH-11-1-0165

TITLE: Targeting Estrogen Receptor-Beta in Triple-Negative Breast Cancer

PRINCIPAL INVESTIGATOR: ~~Chia S. Wu~~ ^

CONTRACTING ORGANIZATION: University of Wisconsin-Madison, Madison WI 53715

REPORT DATE: April 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE: April 2014		2. REPORT TYPE: Annual Summary		3. DATES COVERED 01 Feb 2011 to 31 Jan 2014	
4. TITLE AND SUBTITLE Targeting Estrogen Receptor-Beta in Triple-Negative Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER: W81XWH-11-1-0165	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Erin K. Shanle and Xu, Wei E-Mail: shanle@wisc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF WISCONSIN SYSTEM MADISON, WI 53715-1218				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Estrogen signaling is primarily mediated by two estrogen receptors (ERs), ER α and ER β . Triple negative breast cancer (TNBCs) is an aggressive breast cancer subtype that lacks expression of several therapeutic targets. Based on in vitro and clinical data, it is hypothesized that estrogen receptor (ER) β could be targeted with selective ligands to inhibit the growth of TNBCs. The goal of the work completed over the course of this training program aimed to better understand the role of ER β in TNBC and develop tools to target and detect ER β in TNBCs. First, reporter cell lines with inducible ER α or ER β expression and an estrogen responsive luciferase reporter were developed to identify and characterize subtype selective estrogenic ligands. Second, a tumorigenic TNBC cell line was engineered with inducible ER β expression to determine the effects of ER β on the growth of TNBC cells in vitro and in vivo. These cells were also used to globally identify the ligand dependent and independent ER β target genes using RNA sequencing. Finally, ER β immunohistochemistry was optimized using xenografts and applied to a cohort of TNBCs to assess associations with clinicopathologic features. Not only does this work provide a foundation for further research into the role of ER β in TNBC, it resulted in several publications, presentations, and a rich training experience for a future career in breast cancer research.					
15. SUBJECT TERMS Key words or phrases identifying major concepts in the report					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	62	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	8
References.....	9
Appendix A: Figures and Tables.....	10
Appendix B: Research Manuscripts.....	15

INTRODUCTION

Triple negative breast cancers (TNBCs) account for ~10-15% of breast cancers and are associated with poor clinical outcomes and worse disease-free and overall survival [1]. Thus, there is a critical need to identify new therapeutic targets for this breast cancer subtype. TNBCs lack of expression of three receptors that are commonly used to determine the prognosis and treatment options for patients: estrogen receptor (ER) α , progesterone receptor, and human epidermal growth factor receptor 2 (HER2). A second estrogen receptor (ER β) has been detected in some TNBCs [2-4] and exogenous expression of ER β inhibits the growth of breast cancer cells [5-8]. Given the anti-proliferative activity of ER β , this receptor may be an effective therapeutic target for triple negative breast cancers that express ER β . *The hypothesis underlying this project is that ER β is an effective therapeutic target and ER β selective ligands can inhibit the growth of triple negative breast cancers when ER β is expressed.*

Three aims were originally proposed to test this hypothesis: 1) characterize the growth inhibitory effects of ER β -selective ligands in TNBC cells; 2) identify new ER β -selective ligands using high throughput screening; and 3) determine the effects of ER β -selective ligands on the growth of TNBC in xenograft models. Based on the data collected and feedback from both the committee and the reviewers of the original proposal, some of the original aims and experiments were re-designed and a revised statement of work (SOW) was approved in 2012. This revised SOW incorporated 2 new aims: 1) characterize the growth inhibitory effects of ER β and identify ER β target genes using RNA sequencing (RNA-seq); 2) assess ER β expression in a cohort of TNBC clinical samples. This report will detail the results of each aim from the original SOW and the revised SOW. Key research accomplishments and reportable outcomes will also be described.

BODY

Estrogen signaling is primarily mediated by two estrogen receptors (ERs), ER α and ER β . Despite sharing several structural similarities, ER α and ER β have many unique features with respect to ligand binding and gene regulation. ER α and ER β are expressed in a variety of tissues and have both overlapping and distinct tissue distributions, and there are several ER β isoforms that can modulate the estrogen response. ERs can regulate gene expression in both ligand dependent and independent manners. ER ligands are structurally diverse, and some ligands show selectivity for ER α or ER β due to differences in the ligand binding pockets of the two receptors. Ultimately, ligands can elicit tissue- and cell type-specific responses, as exemplified by selective estrogen receptor modulators (SERMs) such as tamoxifen, which is used to target ER α for breast cancer treatment. ER α is expressed in ~70% of breast cancers, but ~10-15% of breast cancers are classified as triple negative breast cancers (TNBCs). TNBCs lack therapeutic targets and there is a need to identify new treatment strategies for this particularly aggressive breast cancer subtype. ER β is expressed in a subset of TNBCs, and previous *in vitro* data suggested that ER β is growth inhibitory in ER α -negative breast cancers. Based on the results of previous studies, it was hypothesized that ER β may be a therapeutic target in a subset of ER α -negative breast cancers, including some TNBCs.

Aim 1: Characterize the inhibitory effects of ER β -selective compounds in TNBC

The goals of this aim were to: 1) determine the effects of cosmosiin and liquiritigenin on the growth of Hs578T-ER β cells; 2) identify additional models of ER β -positive TNBC; and 3) identify downstream targets of ER β that mediate growth inhibitory effects. Much of the work towards this aim has been published [9, 10] (Appendix B) and was described in the previous annual reports. Briefly, cosmosiin and liquiritigenin inhibited the growth of Hs578T-ER β cells (a TNBC cell line with inducible ER β expression) only when ER β was expressed. This inhibitory effect is likely due to a cell cycle arrest because these compounds did not induce apoptosis (data not shown) and 17 β -estradiol (E2), the most prevalent estrogen in women of reproductive age, was shown to induce a cell cycle arrest in Hs578T-ER β cells [11].

Next, these results were confirmed in another model of ER β -positive TNBC. Because additional models of TNBC with detectable endogenous ER β could not be identified, a tumorigenic TNBC cell line (MDA-468) was engineered to express ER β after doxycycline (Dox) treatment. These MDA468-ER β cells were described in the last annual report, and since then have been described extensively in a recent publication [10] (Appendix B). Briefly, E2 induced a G0/G1 cell cycle arrest in MDA468-ER β cells only when ER β was expressed, and surprisingly, ER β expression alone induced cell cycle arrest, although to a lesser degree. These results suggested that these cells could be used to identify ligand-dependent and ligand-independent ER β target genes in TNBC cells.

In order to identify downstream targets of ER β that mediate growth inhibition, RNA-seq was performed in MDA468-ER β cells in the presence and absence of E2 [10]. This approach allowed the first global identification of ligand-dependent and ligand-independent ER β target genes in the absence of ER α , thereby providing a rich resource for the scientific community. Since this work is published [10], only a few of the results will be highlighted in this report. First, ER β induced the expression of the cyclin dependent kinase inhibitor p21, which mediates the progression of the cell cycle. Second, several common ER β target genes were identified in Hs578T-ER β and MDA468-ER β suggesting that the receptor can mediate a common set of genes in different TNBC cell lines. Finally, in collaboration with a biostatistician, ER β expression was found to be associated with the expression of several of its target genes in TNBC gene expression data from the Cancer Genome Atlas database. Overall, this is the most comprehensive study to date regarding target gene regulation by ER β . In addition, this study allowed me to build a bioinformatics collaboration and develop the skills necessary to perform large-scale genomics studies. These skills will be critical for my future career in breast cancer research.

Original Aim 2: Identify new ER β -selective ligands using high throughput screening (HTS)

The goals of this aim were to: 1) optimize luciferase assays with Hs578T-ER β Luc, 2) screen additional Hs578T-ER α Luc cells, and 3) conduct a screen and counterscreen with small molecule libraries using Hs578T-ER α Luc and Hs578T-ER β Luc cells. Although significant effort went into the high throughput screening optimization, this aim was ultimately deleted because of the variability in the screening assay. As described in the previous annual reports, the assay could not be optimized for a 384 well. However, these reporter cell lines were highly sensitive to estrogenic ligands, and this part of the project ultimately resulted in two first-author publications [9, 12].

Original Aim 3: Determine the effects of ER β -selective ligands on the growth of TNBC in xenograft mouse models

The original goals of this aim were to: 1) graft Hs578T-ER α Luc and Hs578T-ER β Luc cells into nude mice; 2) inject mice with ER β ligands and monitor tumor growth using bioluminescent imaging; and 3) assess luciferase expression and tumor histology after treatments. Although several attempts were made to graft Hs578T-ER α Luc and Hs578T-ER β Luc into nude mice, palpable tumors did not consistently form and this part of the aim was revised.

As an alternative approach, MDA468-ER β cells were labeled with luciferase and injected into the fatpad of nude mice to track the growth of the tumor after treatment with Dox and/or E2. The results of this study are published [10], and the growth inhibitory effects of ER β were confirmed *in vivo*.

Revised Aim 3: Determine the effects of ER β expression and activation on the growth of TNBC in xenograft mouse models and assess ER β expression in TNBC clinical samples

The first part of this aim is described above. In order to address concerns of the reviewers and thesis committee, a second part of the aim was incorporated into the revised statement of work. A major weakness of the original proposal was that it is not widely accepted that ER β is expressed in TNBCs. Many studies aimed at assessing ER β expression have yielded inconsistent results (reviewed in [13]). In order to address the issues of antibody specificity, immunohistochemistry (IHC) protocols for ER β detection were optimized using MDA468-ER β xenograft tissues in which the expression of ER β was regulated by Dox exposure. As shown in Figure 1, ER β was specifically detected in the xenograft tissues obtained from mice exposed to Dox (+ER β) and this signal was eliminated by preabsorbing the antibody with peptide corresponding to ER β . These IHC protocols were then applied to two cohorts of TNBC from the Marshfield Clinic in collaboration with the Translational Research Initiatives in Pathology (TRIP) lab and Dr. Kari Wisinski (manuscript in preparation) (Figure 2). In order to objectively score ER β expression in the samples, the VECTRATM multispectral imaging instrument was used to quantify the ER β signal in the nuclear and cytoplasmic compartments. As shown in Figure 3, there was a close relationship between the percent positive nuclei and the mean optical density (OD) for ER β detection. There was also a close relationship between the cytoplasmic and nuclear ER β expression (Figure 3B and 3C).

The tissues were also stained for Ki67, an indicator of proliferation, to assess whether ER β expression was associated with proliferation. Surprisingly, there was a significant positive correlation between ER β and Ki67 (Figure 4A and 4B). A survival analysis revealed that there was no association between ER β expression and survival in this cohort of TNBCs (Figure 5A and 5B). These results are currently being prepared for publication and will surely be published by the end of the year because this is the first objective, quantitative analysis of ER β expression in TNBC. Although there was a discrepancy between the antiproliferative effects of ER β *in vitro* and the positive association between ER β and Ki67 in clinical samples, this part of the project provides a foundation on which ER β IHC should be performed in future studies with larger cohorts. In addition, this aspect of the project provided experience in bridging basic and clinical research that is necessary for successful translational research. I learned the challenges associated with collaborating and communicating with clinical, statistical and basic researchers, and this invaluable experience will help me successfully drive these translational projects in the future.

KEY RESEARCH ACCOMPLISHMENTS:

- Characterization of growth inhibitory effects of cosmosiin and liquiritigenin in Hs578T-ER β cells
- Generation of MDA468-ER β inducible cell lines
- Characterization of the sensitivity and selectivity of Hs578T-ER β Luc and Hs578T-ER α Luc cells
- Optimization of HTS assay for Hs578T-ER β Luc in 96 well format
- Determined that ER β inhibits MDA468- ER β cell growth by inducing cell cycle arrest
- Demonstrated that ER β regulates p21, a key regulator of G1 arrest
- Developed a retroviral delivery system to express ER β in additional TNBC cell lines
- Identification of ER β targets using RNA sequencing using RSEM and DESeq
- Identified several functions and pathways that are enriched in ER β target genes and validated the ER β target genes using quantitative PCR
- Developed MDA468- ER β -FLuc cells for xenografts experiments and *in vivo* imaging
- Assessed the expression of ER β in a cohort of ER α -negative breast cancer samples
- Determined the associations between ER β expression and clinicopathologic characteristics in a cohort of TNBC

REPORTABLE OUTCOMES:

Conferences Attended:

Society of Toxicology Annual Meeting, 2012

Gordon Research Conference on Hormone Action in Development and Cancer, 2011

Awards:

RSESS Society of Toxicology 2012 Annual Meeting Travel Award, 2012

Publications:

Shanle E, Onitilo A, Huang W, Kim K, Zang C, Engel J, Xu W, Wisinski K. Prognostic significance of full length estrogen receptor beta expression in Stage I-III triple negative breast cancer . *In preparation*.

Yarger JG, Babine RE, Bittner M, **Shanle E**, Xu W, Hershberger P, Nye SH, 2012. Structurally similar estradiol analogs uniquely alter the regulation of intracellular signaling pathways. *J Mol Endocrinol*. 50, 43-57.

Sievers C*, **Shanle EK***, Bradfield C, Xu W, 2012. Differential action of monohydroxylated polycyclic aromatic hydrocarbons with estrogen receptors α and β . *Toxicol Sci* [Epub ahead of print]. *Authors contributed equally to this work

Powell E, **Shanle E**, Brinkman A, Li J, Keles S, Wisinski K, Huang W, Xu W. Identification of Estrogen Receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ER α and ER β . *PLoS ONE*, e30993.

Shanle E, Xu W. Function, expression, and detection of estrogen receptor isoforms in normal and malignant tissues, in: Chen, G. (Ed.), *Estrogen Receptors: Mechanisms, Structure and Role in Disease*. Nova Science Publishers Inc., New York, ISBN 978-1-62257-180-2. (Book Chapter)

Shanle E, Xu W. Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. *Biochem Pharmacol* **82**, 1940 (2011).

Shanle E, Xu W. Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. *Chem Res Toxicol* **24**, 6 (2010). (review)

Poster Presentations:

Blanke, K., **Shanle, E.** Exploring Toxicology: Designing learning goals and evaluation strategies for outreach activities; Accepted for oral presentation in the K-12 Toxicology Outreach Activities: Regional Chapter Successes and Resources Informal Specialty Section. Society of Toxicology Annual Meeting, March 13, 2013.

Shanle, E., Xu, W. Stable reporter cell lines for the identification of subtype selective estrogenic ligands. Society of Toxicology Annual Meeting, March 11-15, 2012. San Francisco, CA. Poster Presentation.

Shanle, E., Xu, W. Stable reporter cell lines for the identification of subtype selective estrogenic ligands. Gordon Conference for Hormone Action in Development and Cancer, July 30-August 4, 2011. Bryant University, Smithfield, RI.

Shanle, E., Powell, E., Eastlund, V., Xu, W. Growth inhibitory effects of natural phytoestrogens on breast cancer cells mediated by ER β . Great Lakes Nuclear Receptor Conference, October 22-23, 2010. University of Michigan, Ann Arbor, MI.

CONCLUSION

At the completion of the Department of Defense Breast Cancer Research Program Pre-Doctoral Traineeship, several significant accomplishments were made. Each aim was successfully completed over the course of the training period, resulting in 3 first-author primary publications, one book chapter, two review articles, and an additional first-author primary publication to be submitted soon. I successfully defended my thesis and received numerous post-doctoral research position offers from institutions including MD Anderson Cancer Center, the University of Colorado, and the University of North Carolina – Chapel Hill. This training program ultimately prepared me for an independent research career and enabled me to pursue a competitive postdoctoral fellowship at the University of North Carolina in Chapel Hill.

REFERENCES:

1. Dent R, Hanna WM, Trudeau M, Rawlinson E, Sun P, Narod SA: **Pattern of metastatic spread in triple-negative breast cancer**. *Breast Cancer Res Treat* 2009, **115**(2):423-428.
2. Marotti JD, Collins LC, Hu R, Tamimi RM: **Estrogen receptor-beta expression in invasive breast cancer in relation to molecular phenotype: results from the Nurses' Health Study**. *Mod Pathol* 2010, **23**(2):197-204.
3. Honma N, Horii R, Iwase T, Saji S, Younes M, Takubo K, Matsuura M, Ito Y, Akiyama F, Sakamoto G: **Clinical importance of estrogen receptor-beta evaluation in breast cancer patients treated with adjuvant tamoxifen therapy**. *J Clin Oncol* 2008, **26**(22):3727-3734.
4. Shaaban AM, Green AR, Karthik S, Alizadeh Y, Hughes TA, Harkins L, Ellis IO, Robertson JF, Paish EC, Saunders PT *et al*: **Nuclear and cytoplasmic expression of ER β 1, ER β 2, and ER β 5 identifies distinct prognostic outcome for breast cancer patients**. *Clin Cancer Res* 2008, **14**(16):5228-5235.
5. Helguero LA, Faulds MH, Gustafsson JA, Haldosen LA: **Estrogen receptors alpha (ER α) and beta (ER β) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11**. *Oncogene* 2005, **24**(44):6605-6616.
6. Treeck O, Lattrich C, Springwald A, Ortmann O: **Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells**. *Breast Cancer Res Treat* 2010, **120**(3):557-565.
7. Murphy LC, Peng B, Lewis A, Davie JR, Leygue E, Kemp A, Ung K, Vendetti M, Shiu R: **Inducible upregulation of oestrogen receptor-b1 affects oestrogen and tamoxifen responsiveness in MCF7 human breast cancer cells**. *J Mol Endocrinol* 2005, **34**(2):553-566.
8. Hodges-Gallagher L, Valentine C, Bader S, Kushner P: **Estrogen receptor beta increases the efficacy of antiestrogens by effects on apoptosis and cell cycling in breast cancer cells**. *Breast Cancer Res Treat* 2008, **109**(2):241-250.
9. Shanle EK, Hawse JR, Xu W: **Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands**. *Biochem Pharmacol* 2011, **82**(12):1940-1949.
10. Shanle EK, Zhao Z, Hawse J, Wisinski K, Keles S, Yuan M, Xu W: **Research resource: global identification of estrogen receptor β target genes in triple negative breast cancer cells**. *Mol Endocrinol* 2013, **27**(10):1762-1775.
11. Secreto FJ, Monroe DG, Dutta S, Ingle JN, Spelsberg TC: **Estrogen receptor alpha/beta isoforms, but not betax, modulate unique patterns of gene expression and cell proliferation in Hs578T cells**. *J Cell Biochem* 2007, **101**(5):1125-1147.
12. Sievers CK, Shanle EK, Bradfield CA, Xu W: **Differential action of monohydroxylated polycyclic aromatic hydrocarbons with estrogen receptors α and β** . *Toxicol Sci* 2013, **132**(2):359-367.
13. Speirs V, Shaaban AM: **Role of ER β in clinical breast cancer**. In: *Hormone Receptors in Breast Cancer*. edn.; 2009: 1-20.

Appendix A: Figures and Tables

Figure 1: Xenograft tissues with inducible ER β expression are useful for optimizing ER β IHC. MDA468-ER β cells were injected into the mammary fat pads of nude mice. After tumors formed, mice were treated with either vehicle (1% sucrose) (A, B, C) or Dox (D, E, F). IHC was performed with the PA1-313 ER β antibody (A, D). For controls, the antibody was pre-absorbed with ER β peptide (B, E) or the primary antibody was excluded entirely (C, F). The brown staining that indicates reactivity toward ER β is only observed in tissues from mice exposed to Dox (+ER β) (A). Scale bars = 100 μ m.

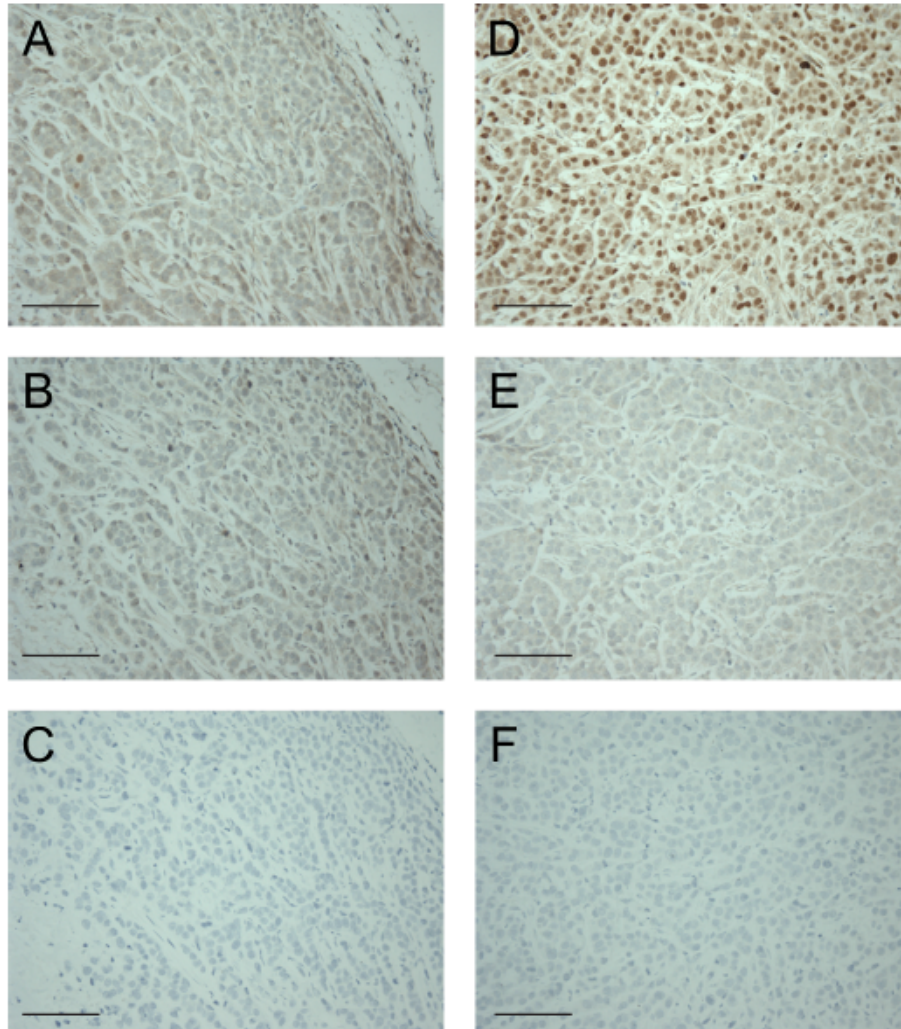


Figure 2: Immunohistochemistry staining for ER β in TNBCs from the Marshfield Clinic. A and B) Representative images of ER β -negative tumor tissues (0.33% and 2.6% 1+ or higher nuclei, respectively). C and D) Representative images of ER β -positive tumor tissues (66.2% and 73.2% 1+ or higher nuclei, respectively). Scale bars = 200 μ m.

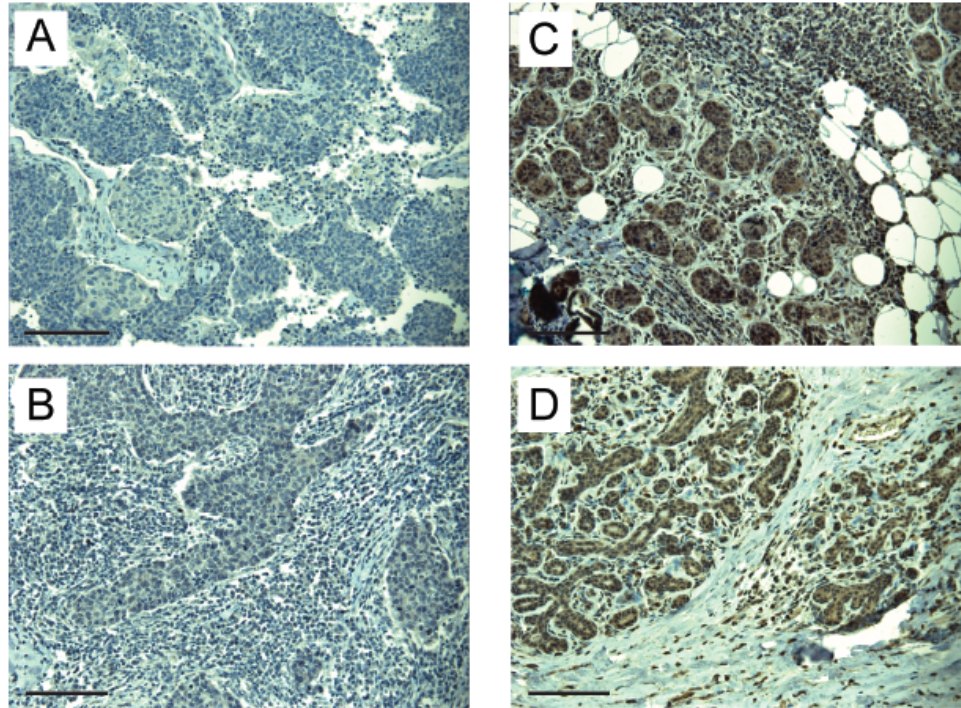


Figure 3: Relationships between ER β subcellular localization and scoring strategies in the Marshfield cohort. A) A comparison of the nuclear mean OD and percent ER β -positive nuclei. B) Relationship between the nuclear and cytoplasmic ER β -positive cells. C) Relationship between the nuclear and cytoplasmic mean OD values.

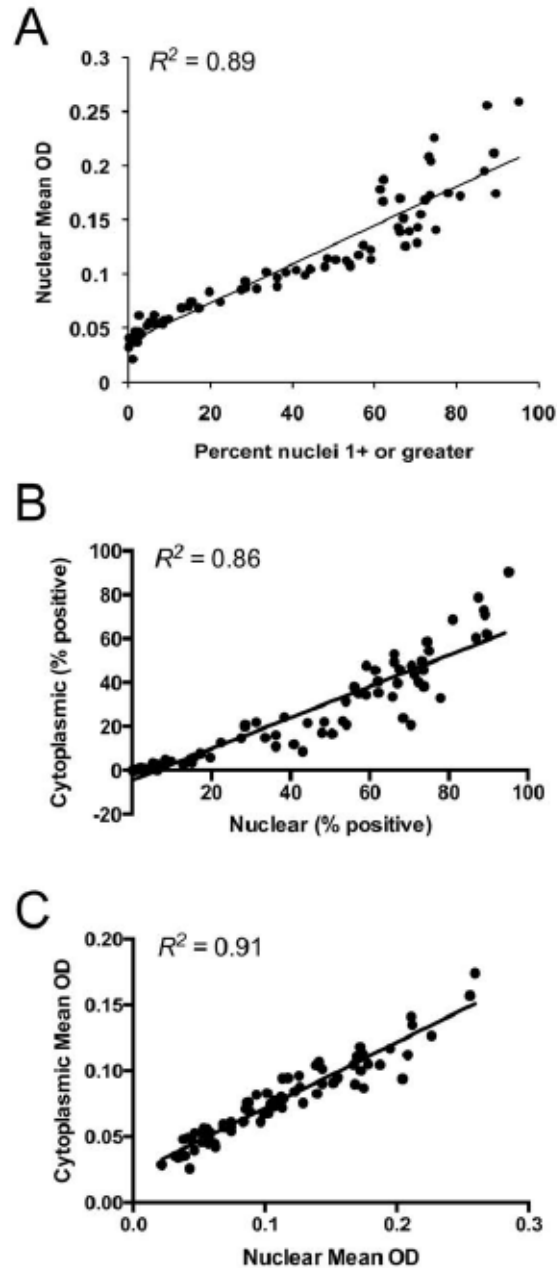


Figure 4: ER β expression is correlated with Ki67 expression. A scatter plot showing the relationship between the percentage of Ki67-positive nuclei (1+ or greater) and the mean OD for (A) nuclear ER β expression or (B) cytoplasmic ER β expression as detected using the PA1-313 antibody. There was a moderately significant positive correlation between the two variables (Spearman correlation, $p < 0.0001$ for both comparisons).

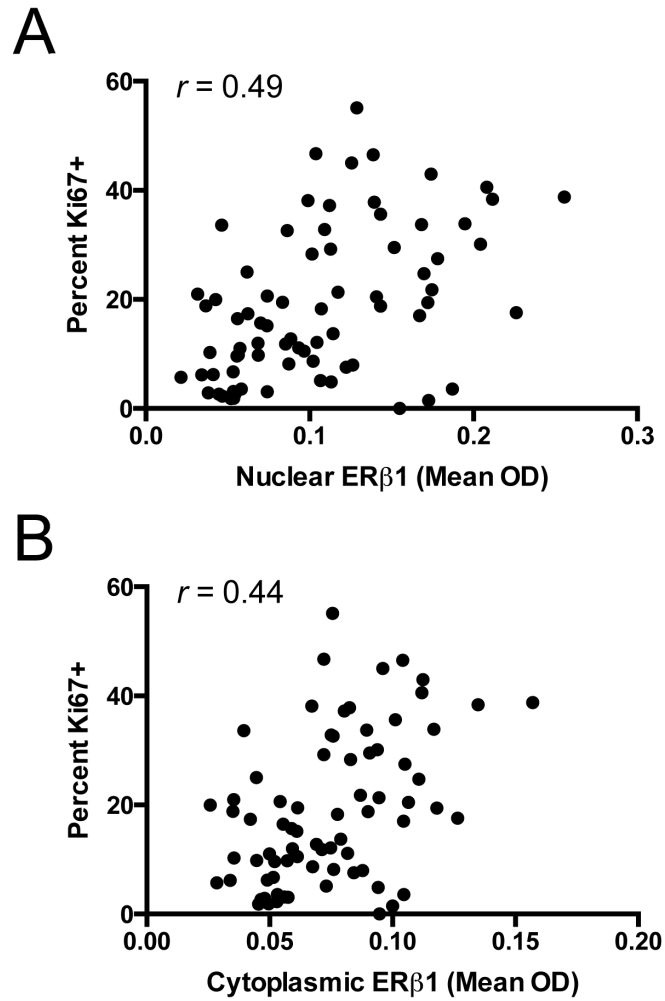
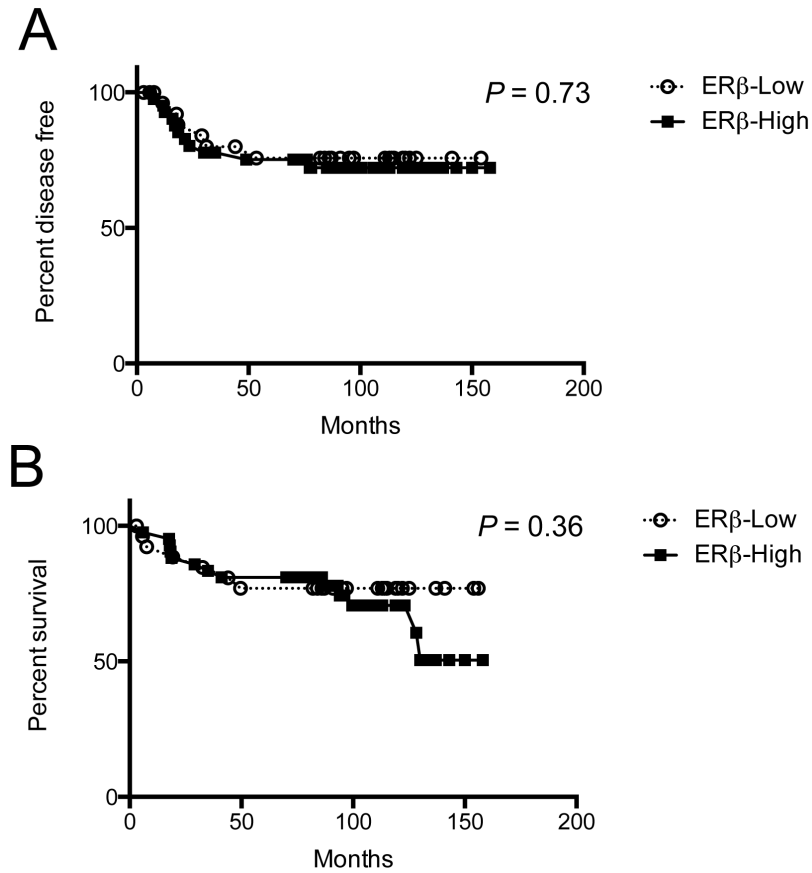


Figure 5: ER β expression is not significantly associated with disease-free survival or overall survival in the Marshfield cohort. Kaplan-Meier graphs for disease-free survival (A) and overall survival (B) with respect to ER β low (<20% nuclear staining, n = 42) and ER β high (>20% nuclear staining, n = 28) expression.



Appendix B: Research Manuscripts

Published in final edited form as:

Biochem Pharmacol. 2011 December 15; 82(12): 1940–1949. doi:10.1016/j.bcp.2011.08.026.

Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands

Erin K. Shanle^{a,b}, John R. Hawse^c, and Wei Xu^{a,b,*}

^aMcArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, USA

^bMolecular and Environmental Toxicology Center, University of Wisconsin, Madison, Wisconsin, USA

^cDepartment of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota, USA

Abstract

Estrogen signaling is mediated by two estrogen receptors (ERs), ER α and ER β , which have unique roles in the regulation of breast cancer cell proliferation. ER α induces proliferation in response to estrogen and ER β inhibits proliferation in breast cancer cells, suggesting that ER β selective ligands may be beneficial for promoting the anti-proliferative action of ER β . Subtype selective ligands can be identified using transcriptional assays, but cell lines in which ER α or ER β are independently expressed are required. Of the available reporter cell lines, none have been generated in breast cancer cells to identify subtype selective ligands. Here we describe the generation of two isogenic breast cancer cell lines, Hs578T-ER α Luc and Hs578T-ER β Luc, with stable integration of an estrogen responsive luciferase reporter gene. Hs578T-ER α Luc and Hs578T-ER β Luc cell lines are highly sensitive to estrogenic chemicals and ER subtype selective ligands, providing a tool to characterize the transcriptional potency and subtype selectivity of estrogenic ligands in the context of breast cancer cells. In addition to measuring reporter activity, ER β target gene expression and growth inhibitory effects of ER β selective ligands can be determined as biological endpoints. The finding that activation of ER β by estrogen or ER β selective natural phytoestrogens inhibits the growth of Hs578T-ER β cells implies therapeutic potential for ER β selective ligands in breast cancer cells that express ER β .

Keywords

Estrogen receptors; subtype selectivity; phytoestrogens; breast cancer

1. Introduction

Estrogens regulate mammary gland growth and differentiation, ovary and uterus maturation, and bone homeostasis [1]. The physiological effects of estrogens are primarily mediated by two estrogen receptors (ERs), ER α and ER β . Because of the broad range of ER target tissues and the ligand dependent activity of the receptors, synthetic and natural estrogens hold

© 2011 Elsevier Inc. All rights reserved.

*Corresponding author: Wei Xu, McArdle Laboratory for Cancer Research, 1400 University Ave, Madison, WI, 53706, United States. Phone: (608) 265-5540; Fax: (608) 262-2824; wxu@oncology.wisc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

therapeutic promise in selectively targeting ERs. Therapies aimed at preventing ER α transcriptional activation are currently used for breast cancer treatment and osteoporosis prevention [2]. Though ER β is not currently a therapeutic target, accumulating evidence suggests an anti-proliferative role for ER β in breast cancer [3]. In the mammary gland, ER α and ER β play opposing roles in regulating growth and differentiation in response to estrogens; ER α promotes proliferation while ER β inhibits ER α -mediated proliferation [4–6]. Because the anti-proliferative action of ER β may be enhanced by ligand-dependent activation, the paradigm of ER targeted therapies is expanding towards the development of ER subtype selective ligands [7].

Though ER α and ER β share many structural and transcriptional features, ligands can display subtype selectivity. In classical ligand dependent transcriptional activation, the receptors dimerize upon ligand binding and undergo conformational changes to allow cofactor recruitment. The receptors directly bind DNA most often at estrogen response elements (EREs), consisting of a consensus GGTCAnnnTGACC sequence. ER α and ER β have 97% identity within the DNA binding domains, and the receptors bind similar DNA sequences with high affinity. Genome wide binding studies in MCF7 breast cancer cells expressing ER α or ER β independently have shown that ER α and ER β bind similar sites in response to 17 β -estradiol (E2); ~60% of ER binding sites contain full EREs and ~25% contain half EREs [8].

The ligand binding pockets of ER α and ER β are relatively large, and the receptors bind a wide array of chemicals. The ligand binding domains of ER α and ER β have 59% identity, and the receptors bind E2 with similar affinities. Despite similarities in their ligand binding domains, several ligands have modest selectivity for ER α or ER β [9], and some synthetic ligands maintain high selectivity. For example, propyl pyrazole triol (PPT) is an ER α selective agonist that displays a 400-fold higher binding affinity for ER α compared to ER β [10]. Estrogenic chemicals produced in plants, known as phytoestrogens, often display subtype selectivity for ER β . For example, liquiritigenin is a flavanone derived from *Glycyrrhizae uralensis* that has been shown to have 20-fold higher binding affinity for ER β and even greater selectivity in transcriptional assays [11]. Compounds such as liquiritigenin often show low binding affinities relative to E2, and ER β selective ligands with higher affinity and greater selectivity are needed to fully elucidate the anti-proliferative role of ER β in breast cancer.

Mammalian cell lines have been developed to enable screening for subtype selective ligands. HeLa cervical carcinoma cells have been used to create HELN-ER α and HELN-ER β , two cell lines in which ER α or ER β , respectively, are constitutively expressed with stable integration of a luciferase reporter downstream of an ERE [12]. Human embryonic kidney cells, HEK293, have also been created using a similar strategy in which ER α or ER β are constitutively expressed and human placental alkaline phosphatase downstream of the vitellogenin ERE is stably integrated [13]. The only available breast cancer reporter cell line is T47D-KBLuc in which three tandem EREs upstream of a luciferase reporter have been stably integrated [14]. However, identification of subtype selective ligands is prohibited because T47D cells express both ER α and ER β .

Here, we describe the generation of two isogenic reporter cell lines, Hs578T-ER α Luc and Hs578T-ER β Luc, that provide a tool to characterize the transcriptional potencies and subtype selectivity of estrogenic compounds in the context of breast cancer cells. These cell lines are highly sensitive to estrogenic ligands and subtype selective ligands and can be used to validate ER transcriptional activation by analysis of endpoints such as endogenous target gene regulation. Further, ER β selective ligands are shown to induce ER β -mediated reporter gene expression, endogenous gene regulation, and growth inhibition, suggesting that

Hs578T-ER β Luc cells may be used to isolate ER β selective ligands with desired biological effects.

2. Materials and Methods

2.1 Cell lines and reagents

Cosmosiin (apigenin 7-glucoside), dimethyl sulfoxide (DMSO), E2, and diethylstilbestrol (DES) were obtained from Sigma (St. Louis, MO); DPN, PPT, and ICI 182,780 were obtained from Tocris (Ellinsville, MO); liquiritigenin was obtained from Chromadex (Irvine, CA). Doxycycline (Dox) was obtained from Clontech. Hygromycin B, blasticidin S, zeocin, NaCl, sodium dodecyl sulfate (SDS), and dithiothreitol (DTT) were obtained from Research Products International (Mount Prospect, IL). Triton X-100 was obtained from Fisher (Fair Lawn, NJ); protease inhibitors were obtained from Roche Scientific (Basel, Switzerland); benzonase was obtained from Novagen (San Diego, CA). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture media were obtained from Invitrogen (Carlsbad, CA). MCF7 and HEK293 cells were cultured in DMEM + 10% fetal bovine serum (FBS; Gemini Bio Products, West Sacramento, CA) at 37 °C and 5% CO₂. Hs578T-ER α and Hs578T-ER β were previously created by Secreto and coworkers [15]. These cells were cultured at 37 °C and 5% CO₂ in DMEM/F12 supplemented with L-glutamine, 10% Tet-system approved FBS (Clontech Mountain View, CA), 500 mg/L Zeocin and 5 mg/L Blasticidin S.

2.2 Generation of Hs578T-ER α Luc and Hs578T-ER β Luc reporter cell lines

Stable reporter cell lines were created using a modified pGL4.32 reporter (Promega, Madison, WI) which contains the *luc2P* reporter and hygromycin resistance. The pGL4.32 vector was digested with *Nhe*I and *Hind*III (New England Biolabs, Ipswich, MA) and three consensus EREs spaced by three nucleotides were cloned upstream of *luc2P* using the following oligonucleotides: 5' –CTA GCG GTC ACA GTG ACC TGC GAG GTC ACA GTG ACC TGC GAG GTC ACA GTG ACC TGC GA – 3' and 5' – AGC TTC GCA GGT CAC TGT GAC CTC GCA GGT CAC TGT GAC CG – 3'. Successful cloning was verified by complete sequencing and the vector was designated pGL4.3xERE. Estrogen responsiveness was validated by batch transfecting HEK293 cells with 2 ng of CMX-ER α or CMX-ER β , 45 ng pGL4.3xERE vector, and 40 ng CMX- β -galactosidase per well of a 48 well plate. Cells were incubated 24 hr to allow protein expression before the addition of the indicated ligands. After 24 hr of ligand treatment, cells were lysed, firefly luciferase substrate (Promega) was added, and luminescence was measured on a Victor X5 microplate reader (Perkin Elmer, Waltham, Massachusetts) using luminescence detection and a 700 nm filter. To normalize data for transfection efficiency, β -galactosidase expression was analyzed using the Tropix β -galactosidase detection kit (Applied Biosystems, Foster City, CA). Luciferase counts were normalized to β -gal counts in each well.

After characterizing the pGL4.3xERE stable reporter vector, Hs578T-ER α and Hs578T-ER β cells were transfected with 10 μ g of the vector and selected in 125 μ g/mL hygromycin B for 4 weeks. Individual colonies were selected using 3 mm cloning discs, expanded, and screened for estrogen induced luciferase expression. One clone from each cell line was selected for further characterization, referred to here as Hs578T-ER α Luc and Hs578T-ER β Luc.

2.3 Quantitative western blots and ligand binding assays

For quantitative western blots, cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox or vehicle (water) 24 hr later. After 48 hr treatment, cells were collected by trypsinization, washed with Dulbecco's phosphate buffer saline (Invitrogen), and lysed by suspension in lysis buffer (50 mM Tris pH 8.0, 400 mM NaCl, 10% glycerol, 0.5% triton X-100, protease inhibitors, and benzonase). After centrifugation, total protein was quantified using BioRad Protein Assay (BioRad), and 40 μ g of protein was resolved using SDS-PAGE and 8% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane for 1.5 hr at 0.35 A. Membranes were blocked with 5% nonfat milk and incubated overnight with 1:1000 anti-FLAG-M2 antibody (Sigma) or 1:5000 anti- β -Actin (Sigma) at 4°C. Membranes were then incubated with IRDye 800CW goat-anti-mouse IgG secondary antibody (Licor Biosciences, Lincoln, NE) for 1 hr at room temperature and visualized on a Licor Odyssey near-infrared gel reader (Licor Biosciences).

For ligand binding assays, Hs578T-ER α Luc and Hs578T-ER β Luc cells were cultured in phenol red free DMEM/F12 + 10% 6x charcoal stripped FBS (SFS) for 3 days prior to the assay to remove residual estrogens from the cells. At 90% confluence, cells were collected, resuspended in phenol red free DMEM/F12 + 5% SFS, and plated at a density of 10^5 cells/well on a 24 well plate in the presence or absence of 50 ng/mL Dox. After 24 hr, cells were labeled in triplicate with 20 nM [3 H]-E2 (89.2 Ci/mmol specific activity, Perkin Elmer) in the presence or absence of 450 μ M DES cold competitor for 2 hr at 37 °C and 5% CO₂. Labeled cells were washed 3 times with cold PBS + 0.1% BSA and lysed with 500 μ L SDS lysis buffer (0.5% SDS, 0.05 M Tris-HCl pH 8.0, and 1 mM DTT). Total cell lysate (400 μ L) was mixed with 5 mL liquid scintillation cocktail and [3 H] bound radioactivity was liquid scintillation counted for 5 min. Two additional wells of each condition were used to count the cell number and determine the total protein using RC DC protein assay (BioRad, Hercules, CA).

2.4 Luciferase assays

Hs578T-ER α Luc and Hs578T-ER β Luc cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were seeded in triplicate at a density of 10^4 cell/well on white 96 well tissue culture plates (Fisher) in phenol red free DMEM/F12 + 5% SFS treated with 50 ng/mL Dox. After 24 hr of Dox treatment, media were replaced with treated media containing vehicle (0.15% DMSO) or a range of serially diluted ligands. All treatments were conducted in the presence and absence of 100 nM ICI 182,780. After treatment for 24 hr, cells were washed with PBS and lysed with 35 μ L lysis buffer (100 mM K₂HPO₄, 0.2% triton X-100, pH 7.8). Lysate (30 μ L) was mixed 1:1 with luciferase substrate (Promega) and luminescence was measured on a Victor X5 microplate reader (Perkin Elmer, Waltham, Massachusetts) using luminescence detection and a 700 nm filter. Total protein (5 μ L) was quantified using BioRad Protein Assay (BioRad). EC₅₀ values were calculated using GraphPad Prism Software (Version 5.04, GraphPad Software Inc., San Diego, CA) and a three parameter log versus response nonlinear regression. Two tailed t-tests performed with GraphPad Prism Software were used to determine statistically significant differences from control treatments.

2.5 Gene expression analysis

For analysis of reporter induction by cosmosiin, Hs578T-ER α Luc and Hs578T-ER β Luc cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox for 48 hr followed by treatment with DMSO (0.1%), 1 nM E2, or 1 μ M cosmosiin for 4 or 24 hr. Total RNA was extracted using RNEasy Plus Kit according to manufacturer protocol (Qiagen, Valencia, CA). RNA (2 μ g) was reverse transcribed using Superscript II RT according to manufacturer protocol (Invitrogen), and firefly luciferase (FLuc) expression

was determined by reverse-transcription polymerase chain reaction using primers shown in Table 1.

For quantitative real-time PCR analysis of endogenous target gene expression, Hs578T-ER α and Hs578T-ER β cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were split in phenol red free DMEM/F12 + 5% SFS and treated with 50 ng/mL Dox for 48 hr prior to ligand treatment. Cells were treated with Dox and ligands or vehicle (0.1% DMSO) for 24 hr, and total RNA was extracted using RNEasy Plus Kit according to manufacturer protocol (Qiagen). RNA (2 μ g) was reverse transcribed as above, and quantitative PCR was performed using TaqMan Prime Time custom designed assays (IDT, Coralville, IA), FastStart Universal Probe Master Mix (Roche Scientific), and a CFX96 instrument (BioRad). Primer and probe sequences are shown in Table 1. Data were analyzed using the $\Delta\Delta$ Cq method calculated by the CFX Manager Software (BioRad). Two tailed t-tests performed with GraphPad Prism Software were used to determine statistically significant differences from control treatments using data from three biological replicates.

2.6 Cell counting assays

Hs578T-ER α and Hs578T-ER β cells were cultured in phenol red free DMEM/F12 + 10% SFS for 3 days prior to the assay to remove residual estrogens from the cells. Cells were seeded at a density of 15,000 cell/well in phenol red free DMEM/F12 + 5% SFS in triplicate in 6 well tissue culture dishes in the presence or absence of 50 ng/mL Dox. After 24 hr, the cells were treated with DMSO (0.1%) or compound in the presence or absence of 50 ng/mL Dox. Media were refreshed every 48 hr, and cells were counted after trypan blue exclusion using an automated cell counter (BioRad) according to manufacturer protocol.

3. Results

3.1 Generation of Hs578T-ER α Luc and Hs578T-ER β Luc reporter cell lines

In order to generate stable reporter breast cancer cell lines, we first cloned a construct encoding a selection marker and a luciferase reporter linked to EREs. The pGL4.32 vector (Promega) contains the *luc2P* gene and was modified to contain 3 tandem consensus EREs upstream of the minimal promoter (pGL4.3xERE, Fig. 1A). Upon complete sequencing, the estrogen responsiveness of the vector was validated in ER-negative HEK293 cells transfected with full length ER α (Fig. 1B) or ER β (Fig. 1C). The pGL4.3xERE reporter showed extremely low background with a 65-fold induction in cells transfected with ER α . The ER antagonist ICI 182,780 abolished estrogen induced expression, reducing the luciferase signal to that of vehicle treated cells. Cells transfected with ER β showed a 15-fold induction of luciferase upon E2 treatment; ICI 182,780 inhibited luciferase expression in both vehicle and estrogen treated cells. The minimal background luciferase expression and the selection marker conferred by the pGL4.3xERE vector made the vector suitable for creating stable reporter cells lines for the identification and characterization of ER selective agonists.

In order to create stable ER reporter breast cancer cell lines, an ER negative breast cancer cell line engineered to express either ER α or ER β was necessary. Previously, Sreter and coworkers created such lines using Hs578T cells [15], a triple negative breast cancer cell line with a basal-like gene expression profile [16]. Hs578T cells lack expression of ER α and ER β providing a clean background in which to express ER α or ER β . Using the tetracycline inducible system, two cell lines were created in which ER α or ER β are inducibly expressed (Hs578T-ER α and Hs578T-ER β cells, respectively) [15]. Hs578T-ER α and Hs578T-ER β cells were transfected with the pGL4.3xERE vector, and individual clones were isolated

after hygromycin selection. Over 20 clones were screened for estrogen induced luciferase expression (data not shown). One clone from each cell line was selected for further characterization, referred to here as Hs578T-ER α Luc and Hs578T-ER β Luc. Additional ER α and ER β reporter clones were used to verify reporter data obtained from Hs578T-ER α Luc and Hs578T-ER β Luc cells.

Hs578T-ER α Luc and Hs578T-ER β Luc cells were first characterized by assessing luciferase induction by ER ligands in the presence or absence of the full antagonist ICI 182,780 (Figure 2). Cells were treated with vehicle, 1 nM E2, 10 nM DPN (a reported ER β selective agonist), or 10 nM PPT (a reported ER α selective agonist). PPT selectively activated luciferase expression in Hs578T-ER α Luc, but DPN activated the reporter in both Hs578T-ER α Luc and Hs578T-ER β Luc cells, though to a lesser extent in Hs578T-ER α Luc cells. Co-treatment with ICI 182,780 blocked luciferase induction in both cell lines (Fig. 2), and luciferase was not induced in the absence of Dox treatment (data not shown).

Basal and E2-induced luciferase signals were much higher in Hs578T-ER α Luc cells when compared to Hs578T-ER β Luc cells, a trend observed in all luciferase assays. On average, Hs578T-ER β Luc cells expressed 630 luciferase units per mg protein and Hs578T-ER α Luc expressed 2900 luciferase units per mg protein at saturating E2 concentrations (0.1 nM or greater). A range of luciferase signals was observed among the clones screened (data not shown), suggesting the accessibility of the reporter in the chromatin may be responsible for differences in luciferase expression. In order to verify Hs578T-ER α Luc and Hs578T-ER β Luc cells had similar ER expression levels at the Dox concentration used throughout the study (50 ng/mL), quantitative western blots were used to compare ER expression in the parent cell lines and reporter cell lines (Fig. 3A). Western blots with FLAG antibody demonstrated similar ER expression in Hs578T-ER α Luc and Hs578T-ER β Luc cells and also confirmed expression levels similar to the parent cell lines. In addition, whole cell ligand binding assays were used to quantify the active receptor in each cell line (Fig. 3B). ER α positive MCF7 breast cancer cells expressed ~150,000 receptors/cell which was very similar to reported values [17]. Both Hs578T-ER α Luc and Hs578T-ER β Luc cells expressed ~120,000 receptors/cell after 50 ng/mL Dox treatment. The comparable number of ERs per cell suggests that differences in ER expression do not account for the higher luciferase signal observed Hs578T-ER α Luc cells. Higher luciferase expression in Hs578T-ER α Luc cells may be due to the accessibility of the reporter in the chromatin or the enhanced transcriptional activity of ER α , in agreement with previous findings that the transcriptional activity of ER α is greater than that of ER β on ERE-containing reporters [18]. Finally, the reporter cell lines did not have an altered morphological phenotype compared to the parent cell lines (Fig. 3C), and no other phenotypic changes due to the integration of the luciferase reporter were observed in Hs578T-ER α Luc and Hs578T-ER β Luc cells.

3.2 Ligand selectivity of Hs578T-ER α Luc and Hs578T-ER β Luc reporter cell lines

We next assessed ligand subtype selectivity using these isogenic reporter cell lines. All luciferase data were normalized to the luciferase signal induced by a saturating concentration of E2 (0.1 nM) and expressed as the percent transactivation relative to 0.1 nM E2. Dose-response curves were obtained for E2, DPN, and PPT to characterize the sensitivity of the reporter cells to ER ligands (Fig. 4). Cells were treated with 10-fold dilutions of ligands and approximate EC₅₀ concentrations for each ligand were calculated from 3 independent experiments (Table 2). The ratios of EC₅₀ values obtained from Hs578T-ER α Luc cells and Hs578T-ER β Luc cells are also presented in Table 2 and provide a measure of the selectivity of the ligands. Higher α/β ratios indicate selectivity for ER β .

Both cell lines were highly sensitive to estrogen (Fig. 4A). Hs578T-ER α Luc cells showed EC₅₀ values near 1 pM; four additional Hs578T-ER α Luc clones showed similar sensitivities

(data not shown). Hs578T-ER β Luc cells also showed EC₅₀ values for estrogen in the pM range, though the average EC₅₀ was 6.5-fold higher than that of Hs578T-ER α Luc cells. Similar differences in estrogen sensitivities have been observed in other ERE-luciferase reporter cell lines expressing ER α or ER β [12–14], suggesting the difference in E2 sensitivity between Hs578T-ER α Luc and Hs578T-ER β Luc cells is due to differences in the transactivation of ER α and ER β .

Next, dose responses to two highly selective ER α and ER β agonists, PPT and DPN respectively, were analyzed using Hs578T-ER α Luc and Hs578T-ER β Luc cells. PPT showed nearly 1000-fold selectivity for ER α (Fig. 4B). Surprisingly, PPT could activate reporter expression in Hs578T-ER β Luc cells at concentrations greater than 100 nM, although it could not induce luciferase expression to the same extent as E2. It has been reported that PPT was unable to induce an estrogen responsive reporter in HEC-1 cells transfected with ER β [10] or in HELN-ER α cells [12]. DPN was not as selective as PPT and could maximally activate luciferase expression Hs578T-ER α Luc cells at 100 nM (Fig. 4C). DPN fully activated ER β at 10 nM. Though DPN has been shown to have a 50 to 70-fold higher binding affinity for ER β [12, 19], comparison of EC₅₀ values showed approximately 30-fold selectivity for ER β in these reporter assays.

Next, the subtype selectivity of two natural phytoestrogens, liquiritigenin and cosmosiin, were analyzed using Hs578T-ER α Luc and Hs578T-ER β Luc cells (Fig. 5). Liquiritigenin is a phytoestrogen derived from *Glycyrrhizae uralensis* and the most active estrogenic component of MF101, an herbal supplement with therapeutic potential [11]. In the initial characterization of liquiritigenin, Mersereau and coworkers found liquiritigenin showed minimal activation of ER α at concentrations up to 2.5 μ M in transcriptional assays in U2OS, HeLa, or WAR5 prostate cancer cells transfected with ER α [11]. Binding assays demonstrated that liquiritigenin had a 20-fold higher affinity for ER β and selectivity was proposed to be due to selective recruitment of co-activators to ER β , namely SRC-2 [11]. Comparison of EC₅₀ values showed liquiritigenin had a 3.6-fold selectivity for ER β , and maximal reporter induction was obtained by 100 nM liquiritigenin in Hs578T-ER β Luc cells and 1 μ M in Hs578T-ER α Luc (Fig. 5A, Table 2).

Cosmosiin, or apigenin 7-glucoside, is a flavone found in chamomile [20] that was identified as an ER agonist that selectively induces ER α / β and ER β / β dimers as measured by bioluminescence resonance energy transfer (BRET) assays (unpublished data). It has a 3-fold higher binding affinity for ER β as measured by competitive ligand binding assays (IC₅₀ ER α 15.9 μ M, IC₅₀ ER β 3.3 μ M, unpublished data). Interestingly, cosmosiin induced luciferase expression to a much greater extent than E2, an effect described as supramaximal induction [21]. Even at concentrations up to 10 μ M, cosmosiin did not saturate the luciferase output, and EC₅₀ values could not be reasonably calculated (Fig. 5B). Another Hs578T-ER β Luc clone treated with cosmosiin also showed supramaximal induction (data not shown). Cosmosiin did not induce luciferase expression in Dox-treated cells co-treated with ICI 182,780 or cells not treated with Dox (data not shown), indicating the supramaximal induction was due to ER β activation. To determine if the supramaximal induction truly represented enhanced transcriptional activation, the transcript levels of luciferase were assessed after 4 and 24 hr treatments of E2 and cosmosiin (Fig. 5C). Cosmosiin did not induce luciferase expression to a greater extent than E2 in either Hs578T-ER α Luc or Hs578T-ER β Luc cells, indicating alternative mechanisms are responsible for the supramaximal effect.

3.3 Selective regulation of ER α and ER β target genes by ER β selective ligands

We next sought to validate the subtype selectivity of DPN, PPT, liquiritigenin and cosmosiin by assessing regulation of endogenous ER target genes. Estrogen responsive target genes of

ER α and ER β were previously identified in Hs578T-ER α and Hs578T-ER β cells [15], and two ER β target genes and one ER α target gene were selected for analysis. Cells were treated with 50 ng/mL Dox for 48 hr to induce expression of the receptors and further treated with the corresponding ligands for 24 hr. Complement component 3 (*C3*, NM_000064) was up-regulated in Hs578T-ER β cells upon E2 treatment (Fig. 6A). DPN and liquiritigenin were capable of inducing *C3* expression to a comparable level as E2 at concentrations that fully activate ER β with minimal ER α activation, as measured by reporter assays (Fig. 6A). Cosmosiin induced *C3* expression at 1 μ M, but not to the same extent as E2, demonstrating cosmosiin does not fully activate the receptor at this concentration. PPT slightly induced *C3* expression compared to DMSO in Hs578T-ER β cells, although PPT induced expression of *C3* to a much lesser degree compared to E2. Repression of the ER β target gene Jagged 1 (*JAG1*, NM_000214) occurred to a similar degree by E2, DPN, liquiritigenin and cosmosiin, although 100 nM liquiritigenin and 1 μ M cosmosiin do not fully repress *JAG1* expression compared to E2, DPN or 1 μ M liquiritigenin (Fig. 6B). Although the ER α selective agonist PPT slightly induced *C3* expression in Hs578T-ER β cells, it had no effect on *JAG1* repression, demonstrating incomplete ER β activation by PPT. To further validate the subtype selectivity observed in reporter assays, expression of the ER α target gene alpha-6 integrin (*ITGA6*, NM_000210) was determined after treatment of Hs578T-ER α cells with E2, DPN, PPT, liquiritigenin and cosmosiin. As shown in Figure 6C, *ITGA6* was up-regulated by E2 and PPT treatment, but DPN and liquiritigenin did not fully activate its expression at concentrations that showed selectivity in reporter assays (10 nM and 100 nM, respectively). At 1 μ M, liquiritigenin and cosmosiin were capable of activating ER α , and *ITGA6* expression was induced in Hs578T-ER α cells.

Therefore, the subtype selectivity of DPN and liquiritigenin observed in reporter cell lines was validated by subtype selective regulation of endogenous target genes. Cosmosiin, however, activated expression of an Hs578T-ER α endogenous gene target at concentrations that only slightly activated luciferase reporter expression in Hs578T-ER α Luc cells.

3.4 Growth inhibition of Hs578T-ER β cells by liquiritigenin and cosmosiin

We next characterized the growth effects of liquiritigenin and cosmosiin in Hs578T-ER α and Hs578T-ER β cells. It was previously shown that E2 inhibits the growth of Hs578T-ER β cells [15], supporting the notion that the anti-proliferative action of ER β may be activated by estrogenic ligands. We tested whether 100 nM liquiritigenin, a concentration at which ER β was selectively activated, and 1 μ M cosmosiin could also inhibit the growth of Hs578T-ER β cells. Hs578T-ER α and Hs578T-ER β cells were treated with vehicle (DMSO), 1 nM E2, 100 nM liquiritigenin or 1 μ M cosmosiin in the presence or absence of 50 ng/mL Dox (with or without ER, respectively) for a total of 5 days. When ER α and ER β were not expressed (-Dox), the compounds had no effect on the growth of the cells (Fig. 7A, B). In contrast, E2, liquiritigenin, and cosmosiin inhibited the growth of Hs578T-ER β cells when ER β was expressed (+Dox, Fig. 7D), and there was an approximately 50% reduction in the number of cells after 5 days of treatment with all three compounds (Fig. 7F). Hs578T-ER α cells showed slight inhibition with E2 and liquiritigenin treatment when ER α was expressed (Fig. 7C), but there was not a statistically significant effect after 5 days of treatment as measured by 2 independent experiments (Fig. 7E). However, ER α expression in ER negative cells often leads to growth inhibition [22, 23], and it is likely that activation of ER α inhibits the growth of Hs578T-ER α cells. This suggests that 100 nM liquiritigenin partially activates ER α despite minimal regulation of *ITGA6* at this concentration.

4. Discussion

ER α is an established therapeutic target for breast cancer treatment, but the development of subtype selective estrogenic ligands has gained interest with the identification of ER β [1].

ER β opposes the actions of ER α suggesting that it may be a potential therapeutic target. Exogenous ER β expression in ER α positive breast cancer cells impaired E2 stimulated proliferation [24] and tumor growth in xenografts [25]. In support of the anti-proliferative role of ER β , MCF7 cells were more proliferative when ER β was knocked down [6]. Activation of ER β by subtype selective ligands may enhance ER β growth repression without stimulating proliferation through ER α ; indeed ER β selective ligands inhibited growth of HC11 mouse mammary cells [5]. Here, we have also shown that ER β ligands can inhibit the growth of breast cancer cells when ER β is expressed. In breast cancer, however, ER β expression is thought to decline during progression [26–28] so ligands aimed at targeting ER β must be highly selective and used only in patients that lack ER α or those with low ER α :ER β ratios of expression. The rate of ER β positivity in breast cancer has been reported to range from 13% to 83% [29–32]. In order to effectively target ER β for cancer treatment, there is an imminent need to: a) identify ER β selective ligands with minimal side effects and better *in vivo* efficacy and selectivity, and b) design clinical trials to recruit patients with low ER α :ER β ratios in earlier stages of disease progression.

Although ER β selective ligands have not yet been used for cancer treatment, the therapeutic value of ER β has been assessed in other diseases. Two of the most promising ER β selective therapies are the ER β selective ligand ERB-041 and the herbal extract MF-101 [33]. Clinical trials have been completed to determine the efficacy of ERB-041 for treatment of Crohn's disease, endometriosis, interstitial cystitis, and rheumatoid arthritis. Although results have not been published for most of the clinical trials, results of the rheumatoid arthritis trial showed ERB-041 was well tolerated but did not improve arthritis symptoms [34]. MF-101 also showed a relatively safe profile and reduced the frequency of hot flashes in a phase II clinical trial for treatment of post-menopausal symptoms [35]. Lignans are the most active estrogenic component of MF-101 [11], suggesting ER β selective ligands may prove useful for treating post-menopausal symptoms.

Strategies to identify ER subtype selective ligands include competitive ligand binding, dimerization, transcriptional reporter, and proliferation assays [21, 36]. Competitive ligand binding assays provide insight into binding affinities and are useful for high throughput small molecule screening [37], but they are limited because ligands can act as agonists or antagonists and binding affinity does not often reflect transcriptional potency. BRET assays to measure receptor dimerization have been used to identify subtype selective ligands [38], but also cannot differentiate between agonists or antagonists [39]. Agonists can be characterized using proliferation assays in MCF7 cells, which are highly sensitive and provide a biologically relevant endpoint in the context of estrogen-sensitive cells [40]. However, this assay is limited by a lack of specificity, as non-estrogenic mitogens can stimulate proliferation, and cannot be used to detect subtype selective agonists.

Transcriptional assays can differentiate between agonists and antagonists, overcoming limitations of binding and dimerization assays. Mammalian reporter cell lines useful for identifying subtype selective ligands have been created from HeLa cervical carcinoma cells [12] and HEK293 kidney cells [13]. HELN-ER α and HELN-ER β were generated from HeLa cells in two steps: 1) stable integration of ERE-luciferase to generate HELN cells, 2) stable expression of ER α or ER β to generate HELN-ER α and HELN-ER β [12]. 293/hER α and 293/hER β cells were generated by a similar strategy. Only one breast cancer reporter cell line, T47D-KBLuc, is available to characterize agonists in the context of breast cancer cells [14], but both ER α and ER β are expressed, preventing identification of subtype selective ligands.

In this report, we described the development of a new set of breast cancer reporter cell lines to characterize subtype selective estrogenic ligands. Hs578T-ER α Luc and Hs578T-ER β Luc cells were highly sensitive to E2 with EC₅₀ values of 1 pM and 6.5 pM, respectively (Fig.

4A). Similar E2 sensitivity was observed in T47D-KBLuc cells, which showed an approximate EC₅₀ of 3 pM [14]. Hs578T-ER α Luc and Hs578T-ER β Luc cells were more sensitive to E2 than HELN-ER and 293/ER reporter cells, but all reporter cell lines showed greater E2 sensitivity in ER α expressing cells. HELN-ER α cells were approximately 3 times more sensitive to E2 than HELN-ER β cells (EC₅₀ of 0.017 nM and 0.068 nM, respectively) [12] and 293/hER α cells were approximately 4 times more sensitive to E2 than cells expressing ER β (EC₅₀ of 50 pM and 200 pM, respectively) [13]. Although Hs578T-ER α Luc and Hs578T-ER β Luc cells were not created using the same strategy as HELN-ER or 293/hER reporter cells and likely have unique genomic integration of the reporter, similar sensitivities observed in all reporter cell lines suggest that this does not inhibit comparison of subtype selectivity.

Reporter assays with two ER subtype selective ligands confirmed that Hs578T-ER α Luc and Hs578T-ER β Luc cells could be used to differentiate between ER α and ER β selective ligands. The ER β selective agonist DPN maintained 33-fold selectivity in Hs578T-ERLuc cells (EC₅₀ of 0.26 nM for ER β and 8.5 nM for ER α , Table 2). Dose response assays with the ER α selective agonist PPT revealed the sensitivity of Hs578T-ER β Luc cells (Fig. 4B). Although PPT was unable to activate reporter expression in HEC-1 cells transfected with ER β [12], PPT did activate reporter expression in Hs578T-ER β Luc cells at high concentrations, although not to the full extent induced by E2. PPT reporter activation was blocked by ICI 182,780 co-treatment (Fig. 2A) and did not occur in the absence of Dox treatment (data not shown), verifying reporter activation was mediated by ER β . Despite activation of ER β at high concentrations, PPT could not fully activate reporter expression in Hs578T-ER β Luc cells and maintained 1000-fold selectivity for ER α .

Subtype selectivity of two natural phytoestrogens, cosmosiin and liquiritigenin, was also assessed in Hs578T-ER α Luc and Hs578T-ER β Luc cells. Liquiritigenin maintained selectivity for ER β but to a lesser extent than expected, as it has been shown to minimally activate ER α in other cell lines [11]. The discrepancy in the selectivity of liquiritigenin may be due to the enhanced sensitivity of Hs578T-ER α Luc cells, differences in cofactor expression in Hs578T cells, or purity of the compound (our studies utilized commercially available liquiritigenin and Mersereau and coworkers [11] used extract from *G. uralensis*). The selectivity of cosmosiin could not be assessed using luciferase assays due to supramaximal induction (Fig. 5B). Supramaximal activation of estrogen responsive reporters have been described in many systems [21]. Here, we showed that supramaximal induction by cosmosiin was not due to enhanced transcriptional activation of the reporter (Fig. 5C). Despite limitations of the reporter system, the subtype selectivity of cosmosiin could be characterized by assessing target gene regulation in Hs578T-ER α and Hs578T-ER β cells. While DPN and liquiritigenin maintained similar extents of selectivity as measured by reporter assays, cosmosiin activated both ER α and ER β as measured by endogenous gene regulation (Fig. 6). Cosmosiin and liquiritigenin induced similar growth inhibitory effects as E2 in Hs578T-ER β cells, indicating the phytoestrogens could elicit ER β activation to a similar extent as E2 (Fig. 7).

Hs578T-ER α Luc and Hs578T-ER β Luc cells have several advantages for identifying ER β selective agonists in comparison to available mammalian reporter cell lines. First, the Hs578T reporter cell lines have inducible expression of ER α and ER β , allowing determination of off-target reporter activation by assessing reporter expression in the absence of Dox. Second, Hs578T-ER α Luc and Hs578T-ER β Luc cells are highly sensitive to estrogenic ligands. Third, endogenous gene regulation can be used to validate subtype selectivity. Finally, growth inhibition assays using Hs578T-ER β cells in the presence and absence of Dox can be used to determine the biological endpoint of ER β activation and validate specificity of ligands to ensure they do not have off-target cytotoxic effects. High

throughput screening may be possible using Hs578T-ER α Luc and Hs578T-ER β Luc cells, and luciferase assay optimization using Hs578T-ER β Luc cells has shown a Z factor of 0.5 (data not shown), an acceptable range for high throughput screening [41]. Therefore, Hs578T-ER α Luc and Hs578T-ER β Luc cells are useful for the identification and characterization of ER subtype selective ligands that may hold therapeutic promise.

Acknowledgments

This work was supported by the National Institute of Environmental Health and Safety (Grant T32 ES007015), the National Institutes of Health (Grants R01CA125387, R03MH089442, CA125387), the Shaw Scientist Award from the Greater Milwaukee Foundation, the Department of Defense Breast Cancer Research Program (Grants BC100252, Era of Hope Scholar Award), and the UWCCC (Multi-IT Grant). We gratefully acknowledge Linda Schuler, Nancy Thompson, and Serife Ayaz-Guner for critical review of the manuscript.

Abbreviations

BRET	bioluminescence resonance energy transfer
Cos	cosmosiin
Dox	doxycycline
DPN	diarylpropionitrile
E2	17 β -estradiol
ER	estrogen receptor
PPT	propyl pyrazole triol
ERE	estrogen response element
ICI	ICI 182,780
Liq	liquiritigenin

References

1. Nilsson S, Gustafsson JA. Estrogen Receptors: Therapies Targeted to Receptor Subtypes. *Clin Pharmacol Ther.* 2011; 89:44–55. [PubMed: 21124311]
2. Swaby R, Sharma C, Jordan V. SERMs for the treatment and prevention of breast cancer. *Rev Endocr and Metab Disord.* 2007; 8:229–39. [PubMed: 17440819]
3. Hartman J, Ström A, Gustafsson JA. Estrogen receptor beta in breast cancer--Diagnostic and therapeutic implications. *Steroids.* 2009; 74:635–41. [PubMed: 19463683]
4. Chang EC, Frasor J, Komm B, Katzenellenbogen BS. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology.* 2006; 147:4831–42. [PubMed: 16809442]
5. Helguero LA, Faulds MH, Gustafsson JA, Haldosen LA. Estrogen receptors alpha (ER α) and beta (ER β) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. *Oncogene.* 2005; 24:6605–16. [PubMed: 16007178]
6. Treeck O, Lattrich C, Springwald A, Ortmann O. Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells. *Breast Cancer Res Treat.* 2010; 120:557–65. [PubMed: 19434490]
7. Minutolo F, Macchia M, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor β ligands: Recent advances and biomedical applications. *Med Res Rev.* 2011; 31:364–442. [PubMed: 19967775]
8. Charn TH, Liu ET-B, Chang EC, Lee YK, Katzenellenbogen JA, Katzenellenbogen BS. Genome-Wide Dynamics of Chromatin Binding of Estrogen Receptors {alpha} and {beta}: Mutual Restriction and Competitive Site Selection. *Mol Endocrinol.* 2010; 24:47–59. [PubMed: 19897598]

9. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*. 1997; 138:863–70. [PubMed: 9048584]
10. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, et al. Pyrazole ligands: Structure-affinity/activity relationships and estrogen receptor- β -selective agonists. *J Med Chem*. 2000; 43:4934–47. [PubMed: 11150164]
11. Mersereau JE, Levy N, Staub RE, Baggett S, Zogric T, Chow S, et al. Liquiritigenin is a plant-derived highly selective estrogen receptor beta agonist. *Mol Cell Endocrinol*. 2008; 283:49–57. [PubMed: 18177995]
12. Escande A, Pillon A, Servant N, Cravedi J-P, Larrea F, Muhn P, et al. Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta. *Biochem Pharmacol*. 2006; 71:1459. [PubMed: 16554039]
13. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J-Å, Nilsson S. Differential Response of Estrogen Receptor α and Estrogen Receptor β to Partial Estrogen Agonists/Antagonists. *Mol Pharmacol*. 1998; 54:105–12. [PubMed: 9658195]
14. Wilson VS, Bobseine K, Gray LE Jr. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. *Toxicol Sci*. 2004; 81:69–77. [PubMed: 15166400]
15. Secreto FJ, Monroe DG, Dutta S, Ingle JN, Spelsberg TC. Estrogen receptor alpha/beta isoforms, but not betacx, modulate unique patterns of gene expression and cell proliferation in Hs578T cells. *J Cell Biochem*. 2007; 101:1125–47. [PubMed: 17520659]
16. Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, et al. Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. *PLoS One*. 2009; 4:e6146. [PubMed: 19582160]
17. Br  nner N, Boulay V, Fojo A, Freter CE, Lippman ME, Clarke R. Acquisition of hormone-independent growth in MCF-7 cells is accompanied by increased expression of estrogen-regulated genes but without detectable DNA amplifications. *Cancer Res*. 1993; 53:283–90. [PubMed: 8380254]
18. Cowley SM, Parker MG. A comparison of transcriptional activation by ER α and ER β . *J Steroid Biochem Mol Biol*. 1999; 69:165–75. [PubMed: 10418990]
19. Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor- β potency-selective ligands: Structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem*. 2001; 44:4230–51. [PubMed: 11708925]
20. Srivastava JK, Gupta S. Antiproliferative and apoptotic effects of chamomile extract in various human cancer cells. *J Agric Food Chem*. 2007; 55:9470–8. [PubMed: 17939735]
21. Monta  o M, Bakker EJ, Murk AJ. Meta-analysis of Supramaximal Effects in In Vitro Estrogenicity Assays. *Toxicol Sci*. 2010; 115:462–74. [PubMed: 20176621]
22. Garcia M, Derocq D, Freiss G, Rochefort H. Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. *Proc Natl Acad Sci U S A*. 1992; 89:11538–42. [PubMed: 1454845]
23. Wang W, Smith R, Burghardt R, Safe SH. 17 beta-Estradiol-mediated growth inhibition of MDA-MB-468 cells stably transfected with the estrogen receptor: cell cycle effects. *Mol Cell Endocrinol*. 1997; 133:49–62. [PubMed: 9359472]
24. Hodges-Gallagher L, Valentine C, Bader S, Kushner P. Estrogen receptor beta increases the efficacy of antiestrogens by effects on apoptosis and cell cycling in breast cancer cells. *Breast Cancer Res Treat*. 2008; 109:241–50. [PubMed: 17638070]
25. Str  m A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor β inhibits 17 β -estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci U S A*. 2004; 101:1566–71. [PubMed: 14745018]
26. Bardin A, Boulle N, Lazennec G, Vignon F, Pujol P. Loss of ER{beta} expression as a common step in estrogen-dependent tumor progression. *Endocr Relat Cancer*. 2004; 11:537–51. [PubMed: 15369453]

27. Shaw JA, Udokang K, Mosquera JM, Chauhan H, Jones JL, Walker RA. Oestrogen receptors alpha and beta differ in normal human breast and breast carcinomas. *J Pathol.* 2002; 198:450–7. [PubMed: 12434414]
28. Skliris GP, Munot K, Bell SM, Carder PJ, Lane S, Horgan K, et al. Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. *J Pathol.* 2003; 201:213–20. [PubMed: 14517838]
29. Marotti JD, Collins LC, Hu R, Tamimi RM. Estrogen receptor-[beta] expression in invasive breast cancer in relation to molecular phenotype: results from the Nurses' Health Study. *Mod Pathol.* 2009; 23:197. [PubMed: 19898422]
30. Honma N, Horii R, Iwase T, Saji S, Younes M, Takubo K, et al. Clinical importance of estrogen receptor-beta evaluation in breast cancer patients treated with adjuvant tamoxifen therapy. *J Clin Oncol.* 2008; 26:3727–34. [PubMed: 18669459]
31. O'Neill PA, Davies MP, Shaaban AM, Innes H, Torevell A, Sibson DR, et al. Wild-type oestrogen receptor beta (ERbeta1) mRNA and protein expression in Tamoxifen-treated post-menopausal breast cancers. *Br J Cancer.* 2004; 91:1694–702. [PubMed: 15477865]
32. Mann S, Laucirica R, Carlson N, Younes PS, Ali N, Younes A, et al. Estrogen receptor beta expression in invasive breast cancer. *Hum Pathol.* 2001; 32:113–8. [PubMed: 11172304]
33. Mohler ML, Narayanan R, Coss CC, Hu K, He Y, Wu Z, et al. Estrogen receptor β selective nonsteroidal estrogens: seeking clinical indications. *Expert Opin Ther Pat.* 2010; 20:507–34. [PubMed: 20302450]
34. Roman-Blas JA, Castañeda S, Cutolo M, Herrero-Beaumont G. Efficacy and safety of a selective estrogen receptor β agonist, ERB-041, in patients with rheumatoid arthritis: a 12-week, randomized, placebo-controlled, phase II study. *Arthritis Care Res (Hoboken).* 2010; 62:1588–93. [PubMed: 20556817]
35. Grady D, Sawaya GF, Johnson KC, Koltun W, Hess R, Vittinghoff E, et al. MF101, a selective estrogen receptor beta modulator for the treatment of menopausal hot flashes: a phase II clinical trial. *Menopause.* 2009; 16:458–65. [PubMed: 19182698]
36. Shanle EK, Xu W. Endocrine Disrupting Chemicals Targeting Estrogen Receptor Signaling: Identification and Mechanisms of Action. *Chem Res Toxicol.* 2010; 24:6–19. [PubMed: 21053929]
37. Bolger R, Wiese TE, Ervin K, Nestich S, Checovich W. Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environ Health Perspect.* 1998; 106:551–7. [PubMed: 9721254]
38. Powell E, Huang SX, Xu Y, Rajski SR, Wang Y, Peters N, et al. Identification and characterization of a novel estrogenic ligand actinopolymorphol A. *Biochem Pharmacol.* 2010; 80:1221–9. [PubMed: 20599778]
39. Powell E, Xu W. Intermolecular interactions identify ligand-selective activity of estrogen receptor a/b dimers. *Proc Natl Acad Sci U S A.* 2008; 105:19012–7. [PubMed: 19022902]
40. Silva E, Scholze M, Kortenkamp A. Activity of xenoestrogens at nanomolar concentrations in the E-Screen assay. *Environmental Health Perspectives.* 2007; 115:91–7. [PubMed: 18174956]
41. Zhang JH, Chung TD, Oldenburg KR. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.* 1999; 4:67–73. [PubMed: 10838414]

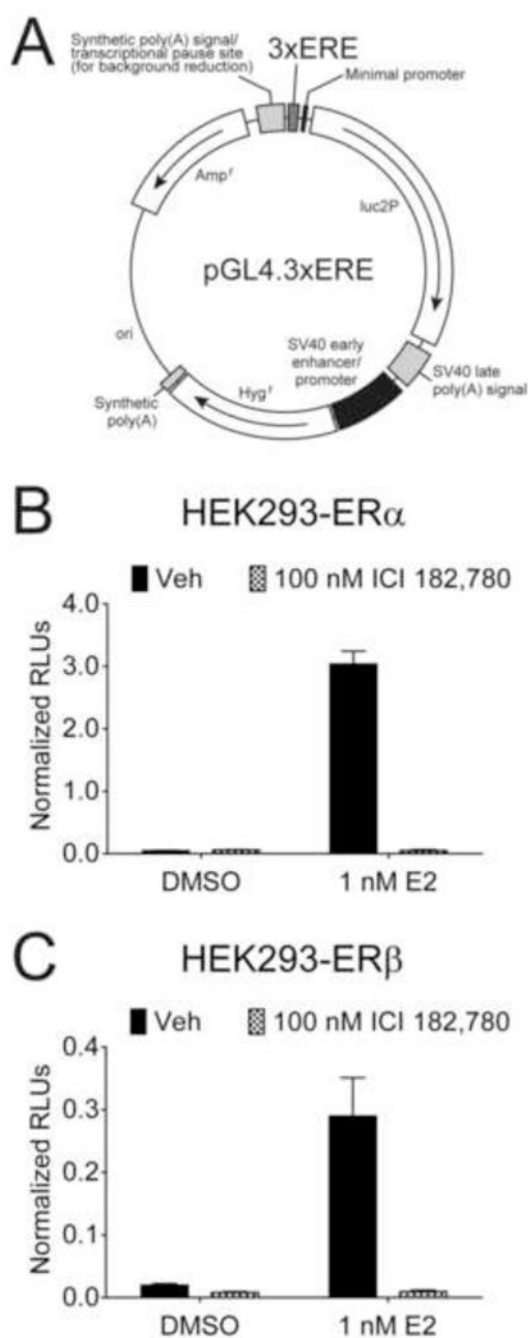


Figure 1.

The pGL4.3xERE reporter construct is estrogen responsive. (A) Three tandem EREs were inserted upstream of the *luc2P* gene in the pGL4.32 luciferase reporter construct. HEK293 cells were batch transfected with the pGL4.3xERE reporter construct, a β -galactosidase construct, and full length ER α (B) or ER β (C). After allowing 24 hr for protein expression, cells were treated in triplicate with vehicle (DMSO) or 1 nM E2 and vehicle or 100 nM ICI 182,780 (0.15 % final DMSO concentration) for an additional 24 hr. Raw luciferase units (RLUs) were normalized to β -galactosidase to normalize for transfection efficiency. Error bars represent standard deviations.

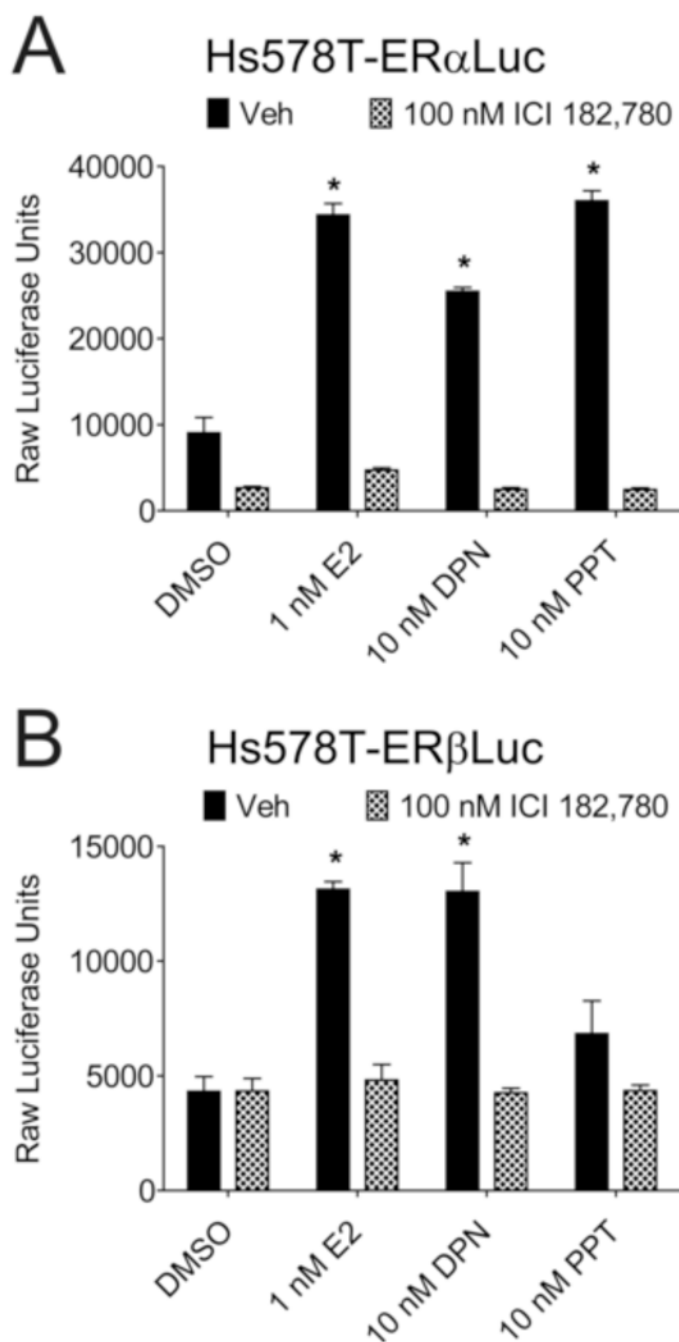
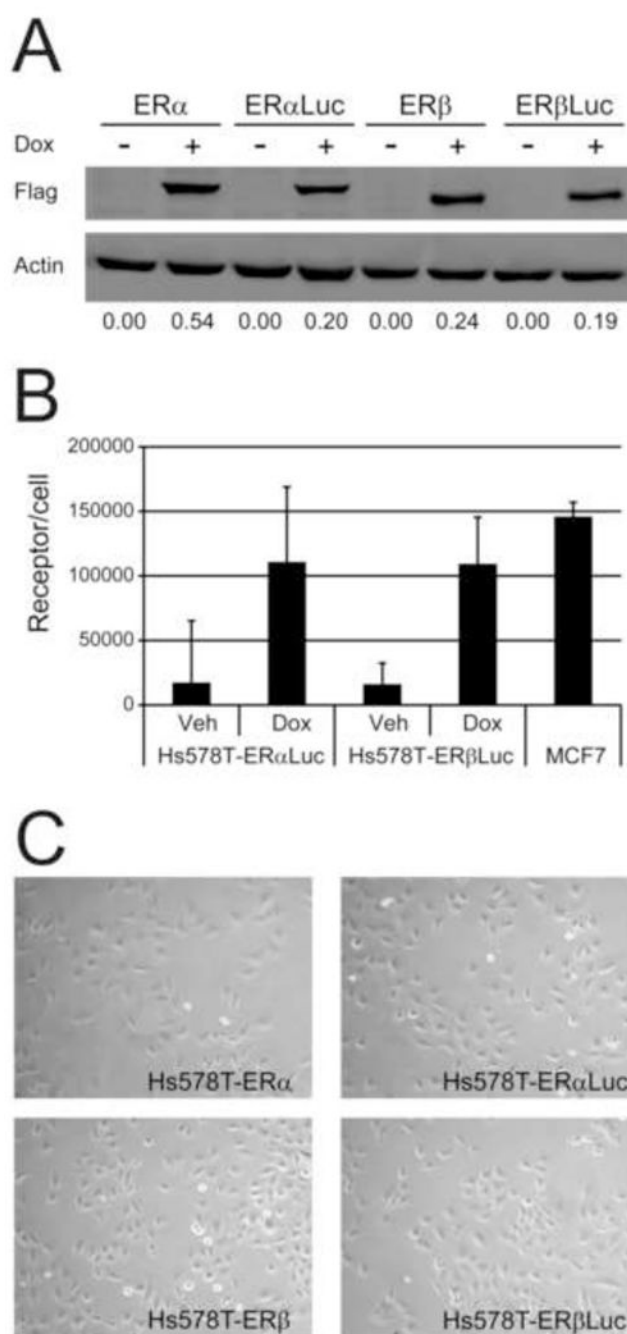


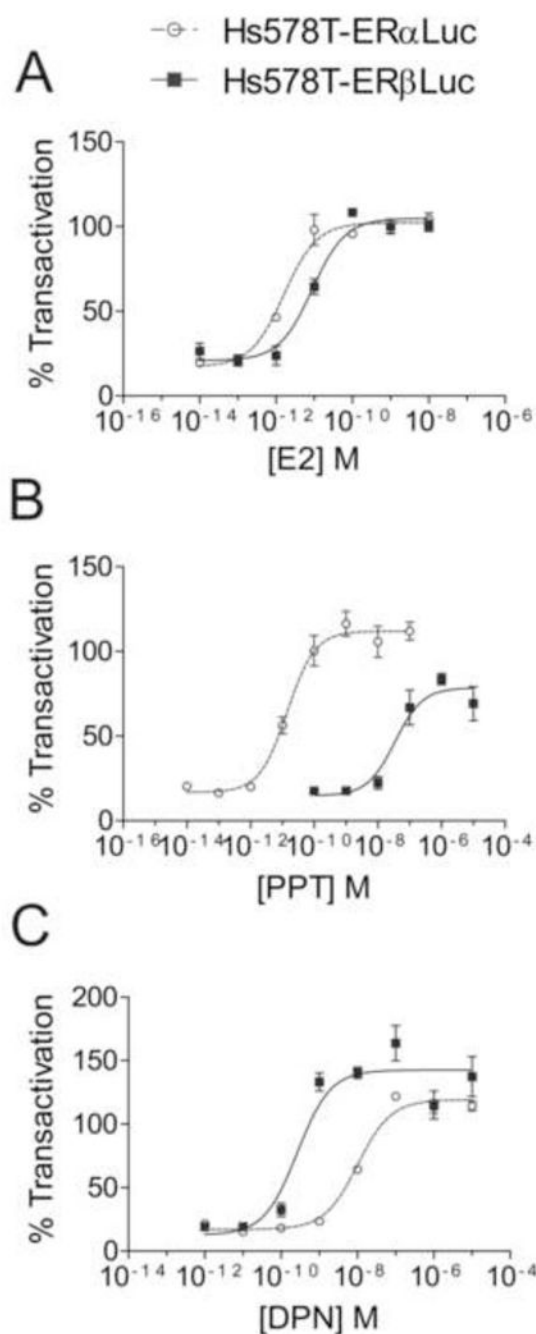
Figure 2.

ER subtype selective ligands selectively induce luciferase in Hs578T-ER α Luc and Hs578T-ER β Luc cells. Hs578T-ER α Luc (A) and Hs578T-ER β Luc (B) cells were seeded in triplicate on 96 well plates in the presence of 50 ng/mL Dox to induce ER expression. After 24 hr, cells were treated with vehicle (DMSO), 1 nM E2, 10 nM DPN, or 10 nM PPT in the presence or absence of 100 nM ICI 182,780 (0.15% final DMSO concentration). Cells were lysed 24 hr after ligand treatment and raw luciferase units were counted. Error bars represent standard deviations. * p values < 0.05

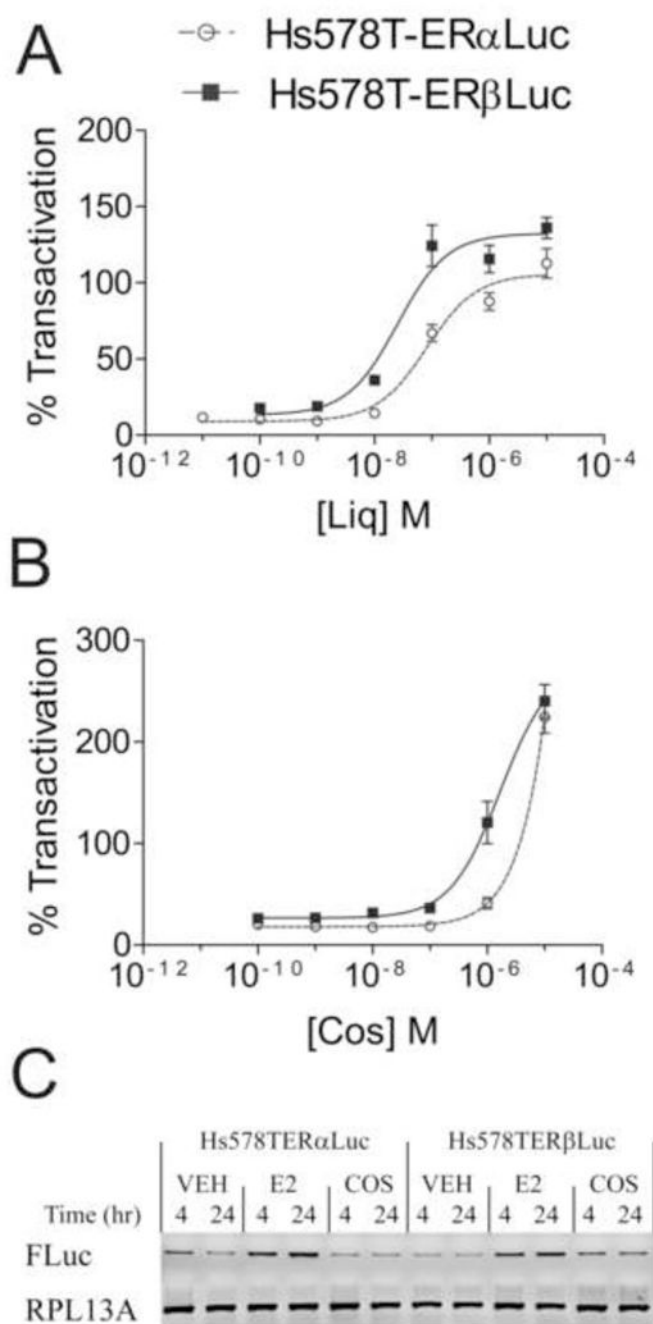
**Figure 3.**

Hs578T-ER α Luc and Hs578T-ER β Luc cells express similar levels of ER. (A) Quantitative western blot with Hs578T-ER α (ER α), Hs578T-ER α Luc (ER α Luc), Hs578T-ER β (ER β), and Hs578T-ER β Luc (ER β Luc) treated with vehicle (-Dox) or 50 ng/mL Dox (+Dox). ER expression was detected using FLAG antibody and quantified by normalizing to b-actin using the Licor Odyssey near-infrared gel reader. The normalized integrated intensity for the FLAG signal is shown below the images. (B) Ligand binding assays confirmed the quantitative western blots. Hs578T-ER α Luc and Hs578T-ER β Luc cells were seeded in triplicate and treated with vehicle or 50 ng/mL Dox for 24 hr. Cells were labeled with 20 nM [3 H]-E2 in the presence or absence of cold competitor for 2 hr, washed, and total cell lysate

was assessed for bound radioactivity as described in Materials and Methods. MCF7 cells were included for comparison. Two additional wells of each cell line and condition were used to determine the cell number and the numbers of receptors per cell were calculated based on a 1:1 molar ratio of ligand to receptor. The average and standard deviation of three independent experiments are shown. (C) The morphology of Hs578T-ER α Luc and Hs578T-ER β Luc was similar to that of the parent Hs578T-ER α and Hs578T-ER β cell lines. Representative phase-contrast microscopy images of each cell line (100X magnification).

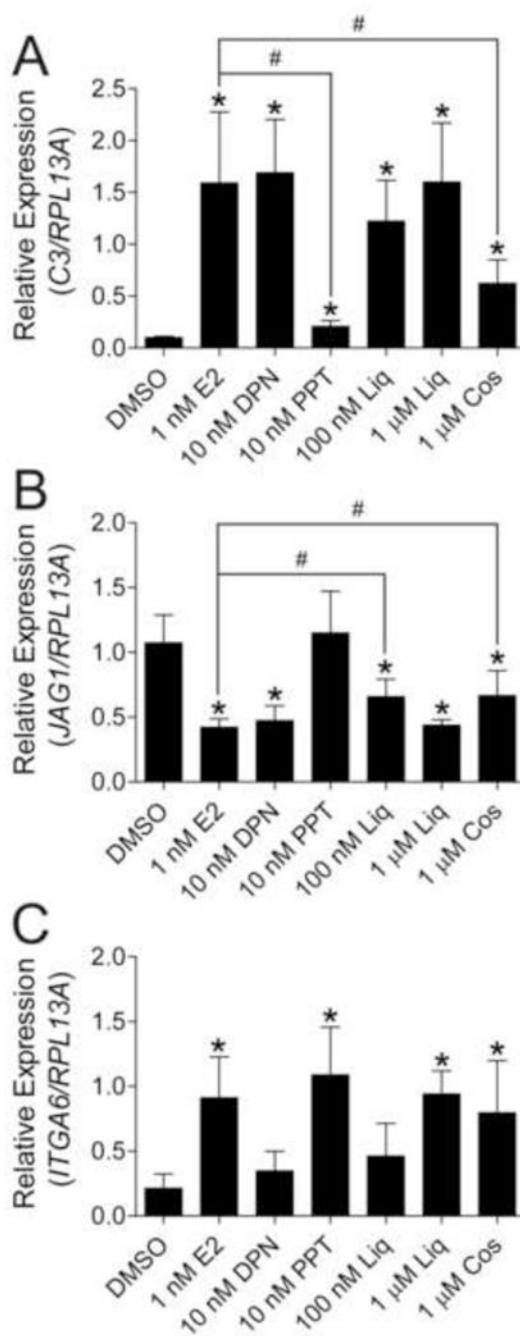
**Figure 4.**

Hs578T-ER α Luc and Hs578T-ER β Luc show subtype selective activation. Dose response curves of E2 (A), PPT (B), and DPN (C). Hs578T-ER α Luc and Hs578T-ER β Luc were seeded in triplicate and treated with 50 ng/mL Dox for 24 hr. Cells were then treated with a range of ligand concentrations (0.15% final DMSO concentration) for 24 hr. Each plate contained DMSO, 0.1 nM E2, and 100 nM ICI 182,780 for controls. Luciferase signal was normalized to total protein in each well and expressed as a percent transactivation relative to signal obtained from saturating E2 treatment (0.1 nM). Each dose response experiment was conducted at least 3 times; data shown are from one representative experiment. EC₅₀ values are shown in Table 2.

**Figure 5.**

Liquiritigenin (Liq) and cosmosiin (Cos) induce reporter expression in Hs578T-ER α Luc and Hs578T-ER β Luc. Dose response curves of liquiritigenin (A) and cosmosiin (B). Hs578T-ER α Luc and Hs578T-ER β Luc were seeded in triplicate and treated with 50 ng/mL Dox for 24 hr. Cells were then treated with a range of ligand concentrations (0.15% final DMSO concentration) for 24 hr. Each plate contained DMSO, 0.1 nM E2 and 100 nM ICI 182,780 for controls. Luciferase signal was normalized to total protein in each well and expressed as a percent transactivation relative to signal obtained from saturating E2 treatment (0.1 nM). Each dose response experiment was conducted at least 3 times; data shown are from one representative experiment. EC₅₀ values are shown in Table 2. EC₅₀ values for cosmosiin

could not be determined because of supramaximal reporter induction. The supramaximal induction by cosmosiin was not due to supramaximal transcription of the luciferase reporter (C). Hs578T-ER α Luc and Hs578T-ER β Luc cells were treated with 50 ng/mL Dox for 48 hr followed by treatment with DMSO (0.1%), 1 nM E2 or 1 μ M cosmosiin for 4 or 24 hr. Firefly luciferase (FLuc) expression was determined by RT-PCR. RPL13A expression was used to ensure equal loading.

**Figure 6.**

ER β selective ligands selectively regulate ER target genes. Hs578T-ER α and Hs578T-ER β cells were treated with 50 ng/mL Dox for 48 hr to induce ER expression followed by treatment with the corresponding ligands for 24 hr. Total RNA was assayed for expression of the ER β target genes *C3* and *JAG1* in Hs578T-ER β cells (A, B respectively) and the ER α target gene *ITGA6* in Hs578T-ER α (C) cells by quantitative reverse-transcription polymerase chain reaction. Target gene expression was calculated using the $\Delta\Delta C_q$ method by normalizing to the ribosomal protein *RPL13A*. Data represent the average and standard deviation of three biological replicates. * p values < 0.05 compared to DMSO control, # p values < 0.05 compared to E2 treatment

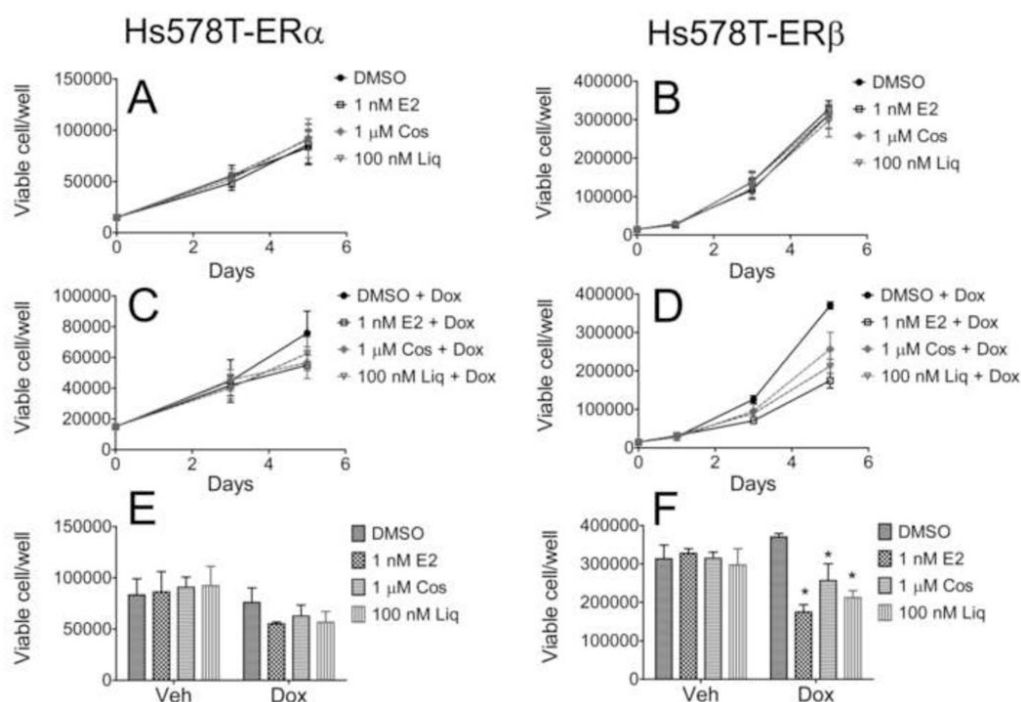


Figure 7.

Cosmosiin (Cos) and liquiritigenin (Liq) inhibit the growth of Hs578T-ER β cells. Hs578T-ER α (A, C, E) and Hs578T-ER β cells (B, D, F) were seeded in 6 well plates and treated with vehicle (A, B) or 50 ng/mL Dox (C, D). After 24 hr, the cells were treated with vehicle (0.1% DMSO) or the indicated ligands, and treatments were refreshed every 48 hr. Cells were counted at the times indicated using trypan blue exclusion. Comparisons of the cell number on day 5 are represented in panels E (Hs578T-ER α) and F (Hs578T-ER β). Data represent two independent experiments. * p values < 0.05

Table 1**Primer and Probe Sequences**

RPL13A	Primer 1	5' - TGT TTG ACG GCA TCC CAC - 3'
	Primer 2	5' - CTG TCA CTG CCT GGT ACT TC - 3'
	Probe	5' - CTT CAG ACG CAC GAC CTT GAG GG - 3'
C3	Primer 1	5' - AAC TAC ATC ACA GAG CTG CG - 3'
	Primer 2	5' - AAG TCC TCA ACG TTC CAC AG - 3'
	Probe	5' - CGT TTC CCG AAG TGA GTT CCC AGA - 3'
JAG1	Primer 1	5' - GGA CTA TGA GGG CAA GAA CTG - 3'
	Primer 2	5' - AAA TAT ACC GCA CCC CTT CAG - 3'
	Probe	5' - TCA CAC CTG AAA GAC CAC TGC CG - 3'
ITGA6	Primer 1	5' - ACC CGA GAA GGA AAT CAA GAC - 3'
	Primer 2	5' - CGC CAT CTT TTG TGG GAT TC - 3'
	Probe	5' - TGG GTT GGA AGG GCT GTT TGT CA - 3'
FLuc	Primer 1	5' - GGC TGA ATA CAA ACC ATC GG - 3'
	Primer 2	5' - CTT TCT TGC TCA CGA ATA CGA - 3'

Table 2Average EC₅₀ values for ER Ligands ($M \times 10^{-9}$)

	Hs578T-ERαLuc	Hs578T-ERβLuc	α/β
E2	0.001 (0.0005)	0.0065 (0.008)	0.15
DPN	8.5 (3)	0.26 (0.02)	33
PPT	0.016 (0.001)	26 (21)	0.001
Liquiritigenin	100 (40)	28 (2)	3.6

Differential Action of Monohydroxylated Polycyclic Aromatic Hydrocarbons with Estrogen Receptors α and β

Chelsie K. Sievers,^{*,1} Erin K. Shanley,^{*,†,1} Christopher A. Bradfield,^{*,†} and Wei Xu^{*,†,2}

^{*}McArdle Laboratory for Cancer Research and [†]the Molecular and Environmental Toxicology Center, University of Wisconsin–Madison, Madison, Wisconsin 53706

¹These authors contributed equally to this work.

²To whom correspondence should be addressed at McArdle Laboratory for Cancer Research, University of Wisconsin–Madison, 1400 University Avenue, Madison, WI 53706. Fax: (608) 262-2824. E-mail: wxu@oncology.wisc.edu.

Received July 13, 2012; accepted September 14, 2012

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of widespread environmental pollutants, some of which have been found to be estrogenic or antiestrogenic. Recent data have shown that hydroxylated PAH metabolites may be responsible for the estrogenic effects of some PAHs. The purpose of this study was to investigate the effects of several PAHs, as well as their monohydroxylated metabolites, on estrogen receptors (ERs), ER α and ER β . Three parent PAHs and their monohydroxylated metabolites were each evaluated using transcriptional reporter assays in isogenic stable cell lines to measure receptor activation, competitive binding assays to determine ligand binding, and bioluminescence resonance energy transfer assays to assess dimerization. Finally, the estrogenic effects of the hydroxylated metabolites were confirmed by quantitative real-time PCR of estrogen-responsive target genes. Although the parent PAHs did not induce ER α or ER β transcriptional activity, all of the monohydroxylated PAHs (1-OH naphthanol, 9-OH phenanthrene, 1-OH pyrene) selectively induced ER β transcriptional activity at the concentrations tested, while not activating ER α . Additionally, the monohydroxylated PAHs were able to competitively bind ER β , induce ER β homodimers, and regulate ER β target genes. Although monohydroxylated PAHs appeared to have weak agonist activity to ER β , our results showed that they can elicit a biologically active response from ER β in human breast cancer cells and potentially interfere with ER β signaling pathways.

Key Words: polycyclic aromatic hydrocarbons; estrogen receptors; monohydroxylated metabolites; dimerization; transcription; ligand binding.

Polycyclic aromatic hydrocarbons (PAHs) have been of increasing concern in the human health field due to their widespread dispersion in the environment and the adverse health effects associated with PAH exposure (Baird *et al.*, 2005). Formed through the incomplete combustion of organic compounds, PAHs can be found in charbroiled foods, cigarette smoke, contaminated soil, vehicle exhaust, and in the atmosphere

from the by-products of industrial processes. PAH exposure can have several adverse effects, including carcinogenesis and endocrine disruption.

Although PAHs are a diverse group of chemicals, most are metabolized by cytochrome P450s, a superfamily of enzymes that mediate the oxidation of lipophilic substrates (Anzenbacher, 2001; Bauer *et al.*, 1995; Kim *et al.*, 1998). The diol epoxide PAH metabolites are capable of inducing DNA damage (Baird *et al.*, 2005), and many PAHs have been shown to be carcinogenic (Bauer *et al.*, 1995; Kim *et al.*, 1998). PAHs can also act as endocrine disrupting chemicals by interfering with normal estrogen signaling. Upon monohydroxylation, PAHs can induce estrogenic effects by directly interacting with estrogen receptors (ERs) (Arcaro *et al.*, 1999; Fertuck *et al.*, 2001a,b). These data suggest that the estrogenic effects of PAHs are primarily mediated by the monohydroxylated PAH metabolites.

ERs, members of the nuclear receptor superfamily of transcription factors, exist in two distinct isoforms, α and β . Encoded by separate genes on different chromosomes, ER α and ER β have both overlapping and unique biological functions. The DNA-binding domains share 96% homology, and ERs bind similar estrogen response elements (EREs) to regulate transcription of target genes. The ligand-binding domains (LBDs), containing the hormone-dependent activation function (AF-2) (Tora *et al.*, 1989), have 55% identity and have similar, but not identical, ligand-binding pockets (Pike *et al.*, 1999). Upon ligand binding, the receptors dimerize and bind DNA to initiate transcription of target genes that mediate distinct biological effects. In the presence of estrogen, ER α is a known driver of cell proliferation, especially in breast cancer cells, whereas ER β has been shown to inhibit ER α -mediated cell proliferation (Hartman *et al.*, 2006; Paruthiyil *et al.*, 2004; Trecek *et al.*, 2010).

Given the critical roles ERs play in regulating cell growth in response to estrogens, there has been significant effort put forth to understand and predict the impacts of xenoestrogens on ER

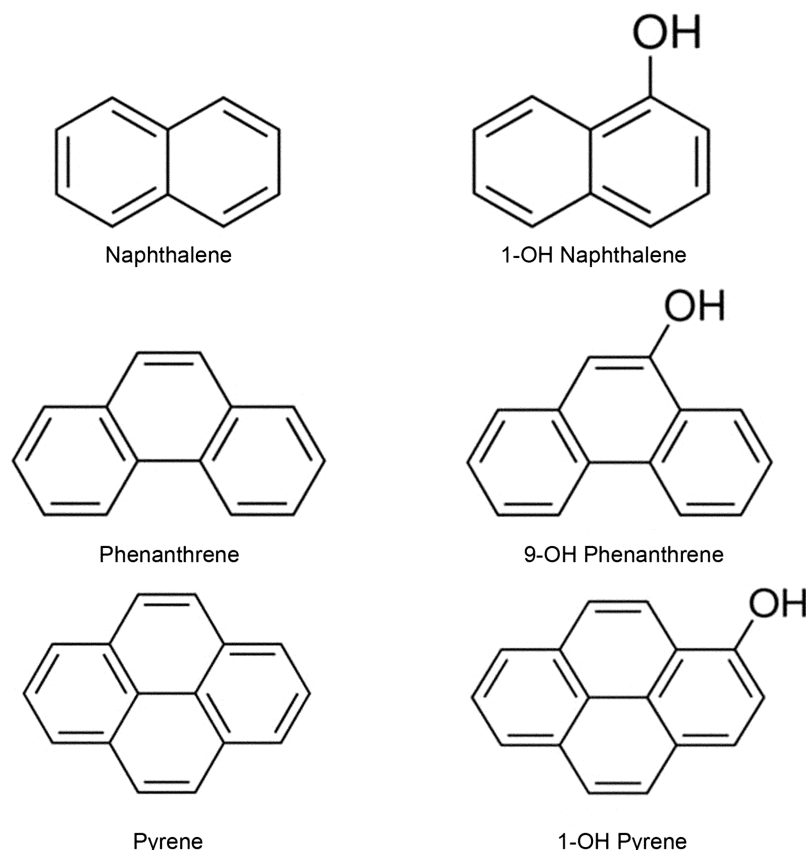


FIG. 1. Chemical structures of select polycyclic aromatic compounds and monohydroxylated metabolites studied.

signaling. However, most studies have been performed solely in the context of ER α , with a limited number of PAHs tested. Here we utilize several *in vitro* assays to assess the effects of three PAHs and their monohydroxylated metabolites, shown in Figure 1, on the transcriptional activation, ligand binding, and dimerization of both ER α and ER β . Compounds were initially screened for transcriptional activation using a previously characterized pair of isogenic breast cancer cell lines with inducible expression of either ER α or ER β and a stably integrated estrogen-responsive reporter (Shanle *et al.*, 2011). These cell lines provide a sensitive tool to directly compare the transcriptional induction of ER α and ER β . Next, bioluminescence resonance energy transfer (BRET) assays were performed to evaluate the dimerization status of ERs. BRET assays are able to monitor protein-protein interactions in a live, cell-based system (Powell and Xu, 2008; Tremblay *et al.*, 1999). Fluorescence polarization experiments were utilized to generate competitive binding curves and determine half maximal inhibitory concentration (IC₅₀) values. This provided a simple, yet specific way to determine whether the tested compound can compete with estrogen for binding to ER. Finally, compounds were evaluated for their ability to upregulate ER β target genes via quantitative real-time PCR (qPCR).

Naphthalene, phenanthrene, and pyrene were chosen as parent PAH compounds for study because they have been detected

at high levels in contaminated environments (Arcaro *et al.*, 1999), and they are considered by to be Priority Pollutants according to the U.S. Environmental Protection Agency. The hydroxylated metabolites were chosen due to their detection after metabolism of the parent compound (Cho *et al.*, 2006; Rossbach *et al.*, 2007). This is the first study to assess ER selective activity of these PAHs and their hydroxylated metabolites at the levels of transcriptional activity using isogenic reporter cell lines, ligand binding, and dimerization. The data demonstrate that monohydroxylated PAHs differentially interact with ER α and ER β and exhibit stronger agonistic activity toward ER β compared with ER α , suggesting that ER β -mediated biological processes need to be evaluated to assess the outcomes of PAH exposure on humans.

MATERIALS AND METHODS

Chemicals. All PAH compounds were purchased from Sigma-Aldrich (St Louis, MO). Doxycycline (Dox) was obtained from Clontech (Mountain View, CA). ICI 182,780 was obtained from Tocris Bioscience (Ellisville, MO).

Cell culture and reporter assays. Cell culture media were obtained from Invitrogen (Carlsbad, CA). HEK293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Gibco Fetal Bovine Serum (FBS; Invitrogen) at 37°C and 5% CO₂. Hs578T-ER α Luc and Hs578T-ER β Luc cells were previously created by Shanle *et al.* (2011) and were

TABLE 1
Primer and Probe Sequences

RPL13A	Primer 1	5'-TGT TTG ACG GCA TCC CAC-3'
	Primer 2	5'-CTG TCA CTG CCT GGT ACT TC-3'
	Probe	5'-CTT CAG ACG CAC GAC CTT GAG GG-3'
C3	Primer 1	5'-AAC TAC ATC ACA GAG CTG CG-3'
	Primer 2	5'-AAG TCC TCA ACG TTC CAC AG-3'
	Probe	5'-CGT TTC CCG AAG TGA GTT CCC AGA-3'
JAG1	Primer 1	5'-GGA CTA TGA GGG CAA GAA CTG-3'
	Primer 2	5'-AAA TAT ACC GCA CCC CTT CAG-3'
	Probe	5'-TCA CAC CTG AAA GAC CAC TGC CG-3'
NR1P1	Primer 1	5'-AGA TTC CCT GTC CTC CTT CA-3'
	Primer 2	5'-GGA AGT GTT TGG ATT GTG AGC-3'
	Probe	5'-TGT GCA TCT TCT GGC TGT GTT TCT CC-3'

maintained in DMEM/F12 supplemented with L-glutamine and 10% Tet-system approved FBS (Clontech) at 37°C and 5% CO₂.

Reporter assays were performed as previously reported (Shanle *et al.*, 2011). Briefly, cells were seeded in triplicate at 10⁴ cells/well on white 96-well tissue culture plates in phenol red-free DMEM/F12 supplemented with 5% charcoal-stripped FBS treated with 50 ng/ml Dox. After 24 h, media were removed and replaced with media treated with 50 ng/ml Dox and vehicle (0.15% dimethyl sulfoxide [DMSO]) or PAH compounds diluted in DMSO. After 24 h of treatment, the cells were washed with 30 μ l of 1 \times PBS and lysed with 35 μ l lysis buffer (100 mM K₂HPO₄, 0.2% Triton X-100, pH 7.8). Thirty microliters of lysate were mixed 1:1 with luciferase substrate (Promega, Madison, WI), and luminescence was measured with a 700-nm filter on a Victor X5 microplate reader (PerkinElmer, Waltham, MA). Total protein was measured using the Bradford Method (Bio-Rad), and raw luciferase data were normalized to total protein. Approximate EC₅₀ values were calculated using GraphPad Prism Software (Version 5.04; Graph-Pad Software Inc., San Diego, CA) and a three-parameter log versus response nonlinear regression.

BRET assays. The BRET assays were performed similarly to those previously reported (Powell and Xu, 2008). Briefly, HEK293T cells were transfected with BRET fusion plasmids (pCMX-ER α -RLuc and pCMX-ER α -YFP or pCMX-RLuc-ER β and pCMX-YFP-ER β). Twenty-four hours after transfection, cells were trypsinized and resuspended in triplicate in PBS at approximately 50,000 cells per well in a white 96-well plate. Cells were then incubated with vehicle (0.6% DMSO), 10 nM E2, or monohydroxylated PAH compound for 1 h at room temperature. Coelenterazine h (Promega) was added to PBS at a final concentration of 5 μ M. Emission measurements at 460 nm and 535 nm were immediately taken on a Victor X5 microplate reader (PerkinElmer). BRET ratios were calculated as previously described (Koterba and Rowan, 2006; Powell and Xu, 2008).

Competitive binding assays. Competitive binding assays were performed using the PolarScreen ER β Competitive Binding Assay Kit, Green (Invitrogen) according to the manufacturer's protocol. Recombinant human ER β (20 nM) and fluorescein-labeled estradiol were incubated for 4 h with the monohydroxylated PAH compounds. Fluorescence polarization was measured using a Victor X5 microplate reader (PerkinElmer). Approximate IC₅₀ values were determined by GraphPad Prism Software (Graph-Pad Software Inc.) from competitive binding curves.

Western blot analysis. Western blots were performed similarly to those previously reported (Shanle *et al.*, 2011) with cells treated for 48 h with vehicle (DMSO) or 10 μ M monohydroxylated PAH compound. Total protein was quantified using Bio-Rad Protein Assay (Bio-Rad), 35 μ g of protein was resolved by SDS-PAGE, and membranes were incubated with 1:1000 anti-FLAG-M2 antibody (Sigma) overnight at 4°C. Membranes were then incubated with goat anti-rabbit HRP secondary antibody (Licor Biosciences, Lincoln, NE) for 1 h at room temperature and visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific, Waltham, MA)

on autoradiography film. Membranes were then washed and incubated with 1:5000 anti- β -Actin (Sigma) for 1 h at room temperature, then incubated with goat anti-mouse HRP secondary antibody (Licor Biosciences) for 1 h at room temperature and visualized using SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) on autoradiography film.

qPCR analysis. Hs578T-ER β Luc cells were cultured in phenol red-free DMEM/F12 supplemented with 10% charcoal-stripped FBS for 3 days prior to experiment to remove any residual estrogens. Cells were seeded into 10-cm tissue culture plates in phenol red-free DMEM/F12 supplemented with 5% stripped serum and treated with 50 ng/ml of Dox 24 h prior to PAH treatment. Cells were then treated with 50 ng/ml Dox plus 0.1% DMSO control, 10 nM E2, 10 μ M 1-OH-naphthalene, 5 μ M 9-OH phenanthrene, or 5 μ M 1-OH pyrene for 24 h. Total RNA was extracted using HP Total RNA Kit (VWR Scientific, West Chester, PA) according to the manufacturer's protocol. One microgram of RNA was reverse transcribed using Superscript II RT according to the manufacturer's protocol (Invitrogen), and qPCR was performed using TaqMan Prime Time custom designed assays (IDT, Coralville, IA), FastStart Universal Probe Master Mix (Roche Scientific, Basel, Switzerland), and a CFX96 instrument (Bio-Rad). Primer and probe sequences are shown in Table 1.

Statistical analyses. Two-tailed Student's *t*-tests were performed using GraphPad Prism version 5.04 for Windows, GraphPad Software (www.graphpad.com).

RESULTS

Monohydroxylated PAHs Selectively Activate ER β in Reporter Cell Lines

In order to test the hypothesis that hydroxylated PAHs may have estrogenic activity with differential effects on ER α and ER β , we first utilized Hs578T-ER α Luc and Hs578T-ER β Luc reporter cells (Shanle *et al.*, 2011). These cell lines have inducible expression of ER α or ER β , respectively, and a stably integrated luciferase reporter just downstream of three tandem EREs. Previous work has shown that these cell lines are highly sensitive to estrogenic ligands and can be used to distinguish ER subtype selective ligands (Shanle *et al.*, 2011). In this system, cells are first treated with Dox to induce expression of the receptor, followed by treatment with the corresponding compounds. In our initial experiments comparing the activation of ER α and ER β , we observed that only hydroxylated PAHs conferred estrogenic activity at 10 μ M (Fig. 2). The monohydroxylated PAH

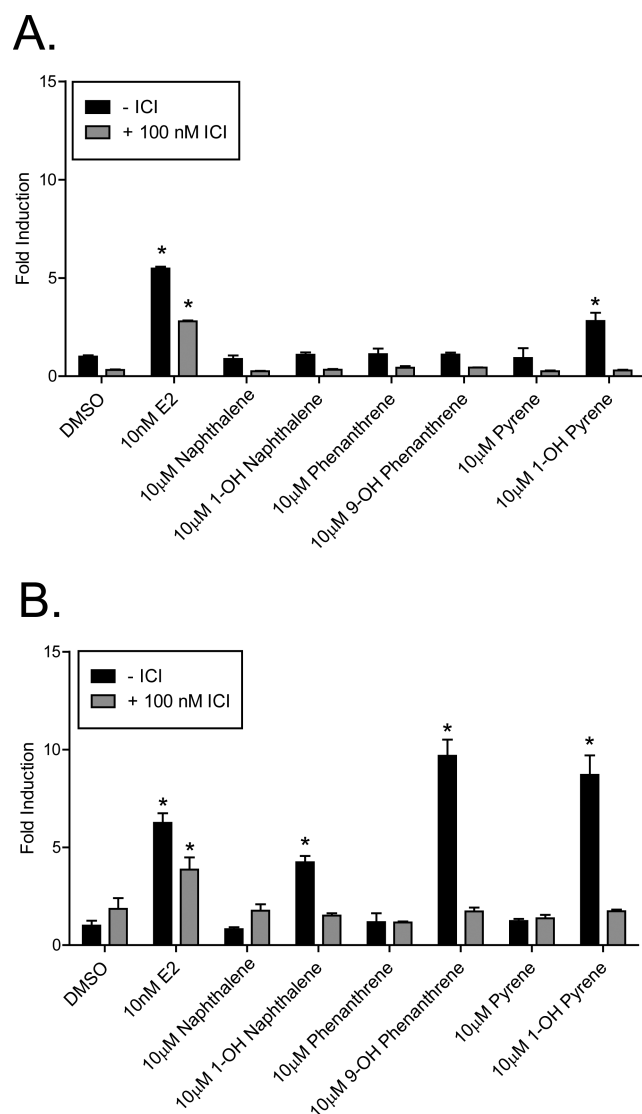


FIG. 2. Differential activation of ER α and ER β by select monohydroxylated PAH compounds. (A) Hs578T-ER α Luc and (B) Hs578T-ER β Luc stable cell lines were treated in triplicate with 10 μ M of PAH compound in the presence or absence of 100nM ICI 182,780 for 24h. Data are expressed as fold induction of raw luciferase units per mg protein over the DMSO control \pm SD. Experiments were repeated at least twice. * p < 0.01 compared with DMSO control.

compounds were able to induce the ERE-luciferase reporter activity primarily in the Hs578T-ER β Luc cells (Fig. 2B). In these cells, 1-OH naphthalene, 9-OH phenanthrene, and 1-OH pyrene induced a 4.2-, 9.7-, and 8.7-fold change over DMSO vehicle control, respectively (p < 0.01 in all cases). In contrast, only 1-OH pyrene induced the ERE-luciferase reporter activity in the Hs578T-ER α Luc cell line (p < 0.01), but not nearly to the same degree as that of 17 β -estradiol (E2) (Fig. 2A). The ER antagonist ICI 182,780 blocked PAH-induced expression in all cases, reducing the luciferase signal to that of vehicle-treated cells. Reporter expression induced by 10nM E2 was not fully blocked by ICI 182,780 cotreatment because of the high

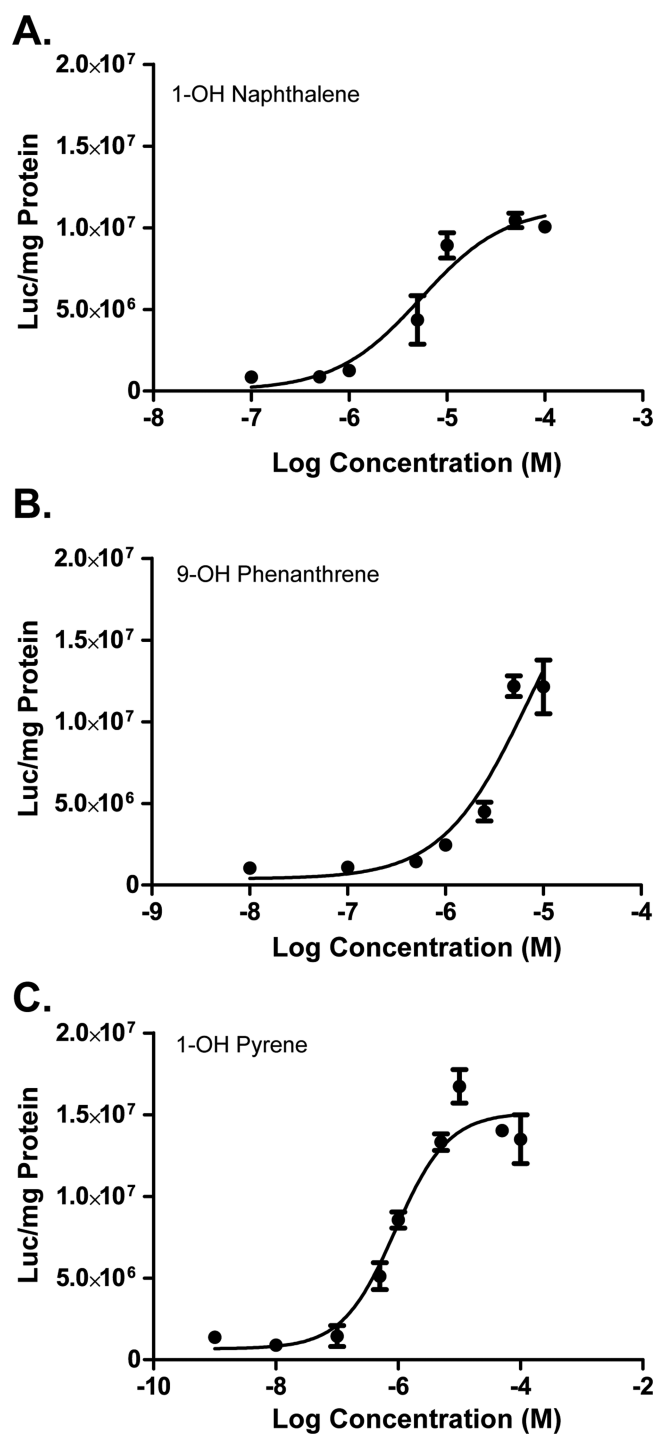


FIG. 3. Monohydroxylated PAHs activate ER β in a dose-dependent manner. Hs578T-ER β Luc cells were treated with Dox for 24h followed by treatment with a range of concentrations of (A) 1-OH naphthalene, (B) 9-OH phenanthrene, or (C) 1-OH pyrene. The mean and SD shown are from triplicates of one representative experiment repeated twice.

concentration and potency of E2. No induction of reporter gene activity was seen in control experiments in which cells were not treated with Dox (Supplementary fig. 1), further confirming ER-mediated induction of the luciferase reporter.

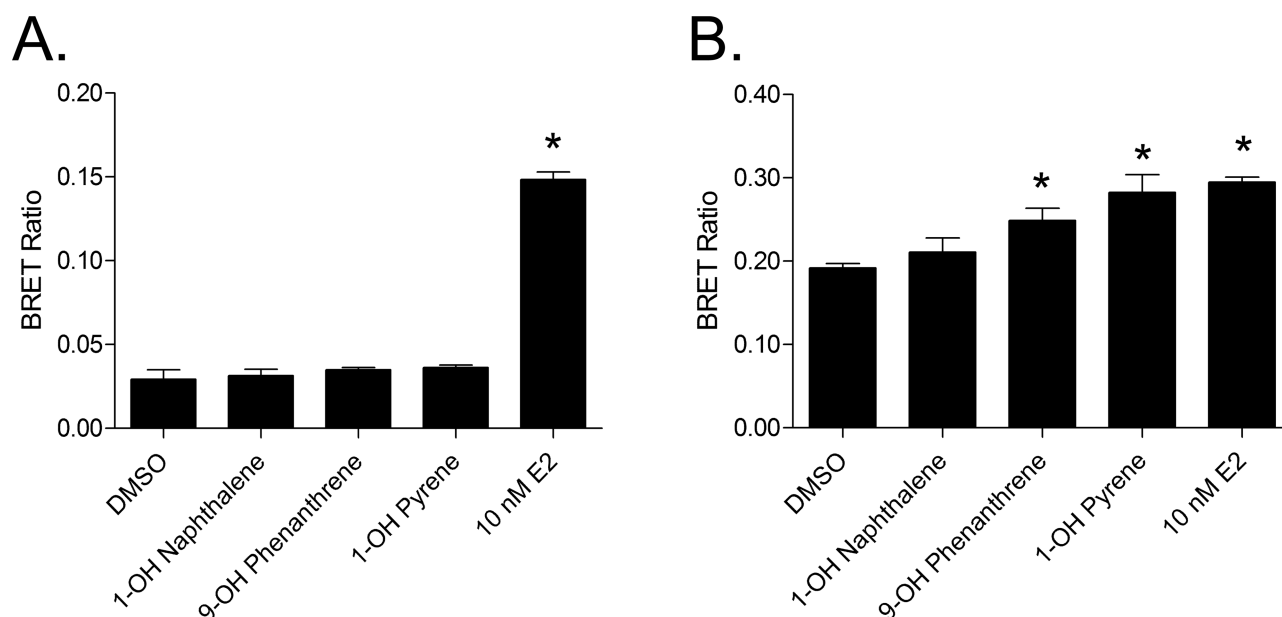


FIG. 4. Monohydroxylated PAH compounds selectively induce ER β /ER β homodimers. (A) BRET data for 293T cells transfected with CMX-ER α -RLuc and CMX-ER α -YFP, showing no ER α /ER α dimerization upon treatment with PAH compounds in triplicate. (B) BRET data for 293T cells transfected with CMX-RLuc-ER β and CMX-YFP-ER β , showing ER β /ER β dimerization when treated with PAH compounds in triplicate. The experiment was performed three times. Error bars represent SEM. * $p < 0.05$ compared with DMSO control.

We next determined the dose-dependent effects of the hydroxylated PAHs in the Hs578T-ER β Luc cells (Fig. 3). The half maximal effective concentration (EC_{50}) values for 1-OH naphthalene and 1-OH pyrene were found to be approximately 5.38 and 0.89 μ M, respectively. 9-OH Phenanthrene proved to be cytotoxic at concentrations greater than 10 μ M, and the dose-response curve did not adequately saturate; however, an approximate EC_{50} value was estimated to be $\geq 6.8 \mu$ M.

Monohydroxylated PAHs Induce ER β Dimers and Directly Bind the Receptor

To further dissect the mechanism through which the monohydroxylated PAHs activate ER β and confirm the selectivity of the compounds, ER dimerization induced by the compounds was assessed using BRET assays. BRET assays allow the determination of dimer formation in live cells by transfecting cells with an energy donor (ER fused to Renilla luciferase) and acceptor (ER fused to yellow fluorescent protein) (see Powell and Xu, 2008). Upon transfecting the cells with the fusion constructs for ER α or ER β , 9-OH phenanthrene and 1-OH pyrene were shown to significantly induce ER β homodimerization ($p = 0.02$ and 0.01 , respectively) (Fig. 4B). In contrast, 1-OH naphthalene did not significantly induce ER β dimerization as determined by the BRET assay ($p = 0.35$). Following the trend seen in the ER α ERE-reporter assay, the monohydroxylated PAH compounds were unable to induce ER α homodimers (Fig. 4A).

In order to confirm that ER β dimerization and ERE-luciferase activity were directly induced by ligand binding, the ability of the monohydroxylated PAH compounds to displace

fluorescein-labeled estradiol from human ER β was assessed in a competitive binding assay (Fig. 5). The competition with E2 indicates that compounds directly bind to ER β in the same ligand-binding pocket as E2. These competitive binding data yielded half maximal inhibitory concentration (IC_{50}) values for 9-OH phenanthrene and 1-OH pyrene at 9.75 and 0.69 μ M, respectively. In support of the BRET results, 1-OH naphthalene showed a much lower affinity for ER β as evidenced by Figure 5A, but it was still able to displace E2 at higher concentrations. The approximate IC_{50} value for 1-OH naphthalene was estimated at or greater than 0.48 μ M.

After determining that monohydroxylated PAHs bind ER β , Western blots with FLAG antibody were used to determine the degradation status of the receptor (Supplementary fig. 2), as some ER ligands cause degradation of the receptor upon binding. These Western blots confirmed that ER β was not degraded by the monohydroxylated PAHs within 48 h of treatment.

Monohydroxylated PAHs Exhibit Estrogenic Activity on ER β Target Genes

To further validate the reporter assay and BRET assay results, the regulation of endogenous ER β target genes was assessed. Estrogen responsive target genes of ER β were previously identified in Hs578T-ER β cells (Secreto *et al.*, 2007). Two upregulated target genes (*CC3* and *NR1P1*) and one downregulated target gene (*JAG1*) were selected for analysis by qPCR (Fig. 6). At 10 μ M, 1-OH naphthalene was able to induce *CC3* and *NR1P1* expression 2.1- and 2.2-fold over DMSO, respectively. Although the increased expression of *CC3* did not reach statistical significance ($p = 0.06$),

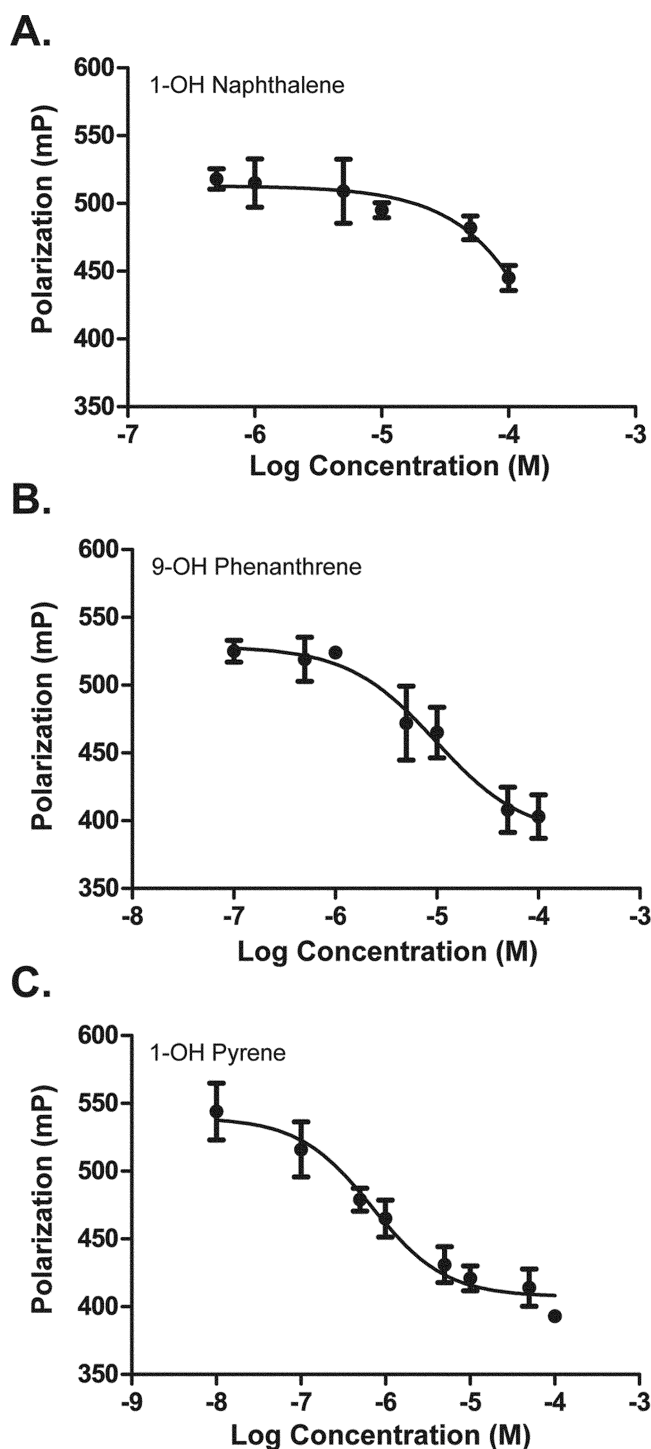


FIG. 5. Monohydroxylated PAHs can bind ER β *in vitro*. Competitive binding curves for monohydroxylated PAH compounds displacing fluorescein-labeled estradiol from human ER β . Purified hER β and fluorescein-labeled estradiol were incubated for 4 h with serial dilutions in triplicate of (A) 1-OH naphthanol, (B) 9-OH phenanthrene, and (C) 1-OH pyrene. Error bars represent SD.

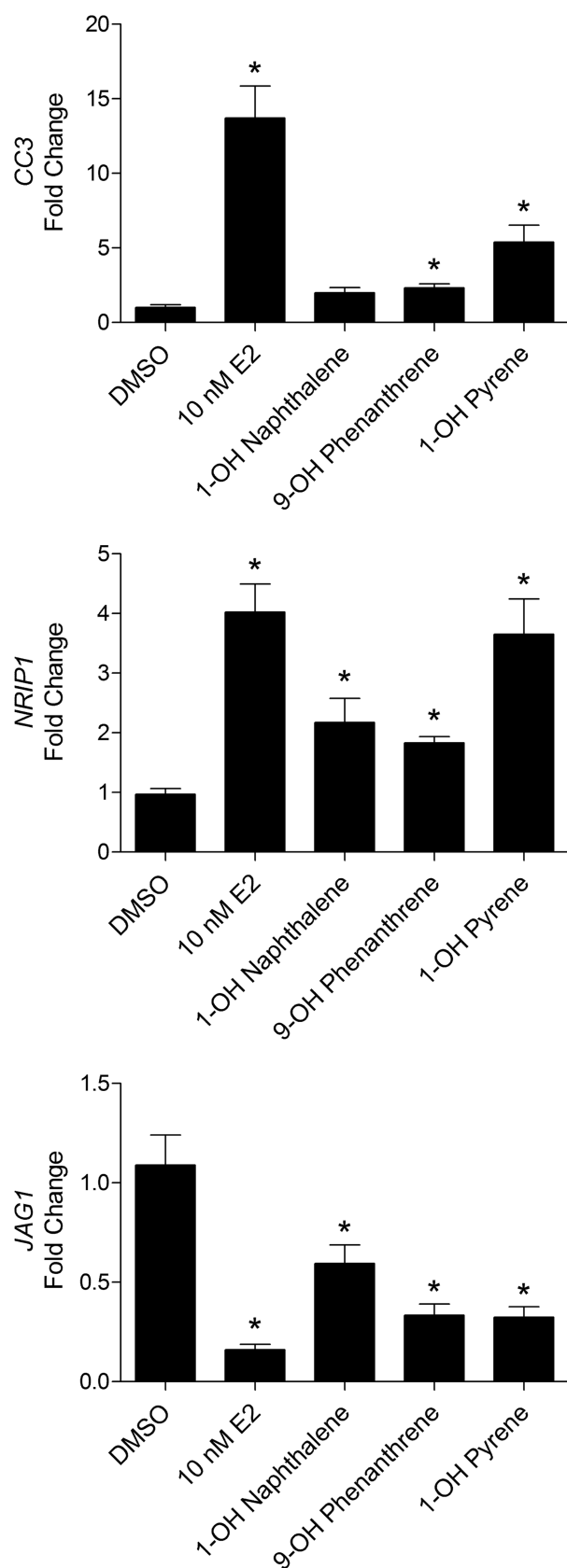
NR1P1 was significantly upregulated by 1-OH naphthalene ($p = 0.02$). Treatment with 5 μ M 9-OH phenanthrene was able to significantly induce *CC3* and *NR1P1* expression 2.4-fold ($p = 0.02$) and 1.9-fold ($p < 0.01$) over DMSO, respectively. Similarly, 5 μ M 1-OH pyrene was able to significantly induce *CC3* and *NR1P1* expression 5.6-fold ($p = 0.02$) and 3.8-fold ($p < 0.01$) over DMSO, respectively. Additionally, all three monohydroxylated PAH compounds were able to downregulate the expression of *JAG1*, generating mean fold changes of 0.64 ($p = 0.02$), 0.36 ($p < 0.01$), and 0.32 ($p < 0.01$) over the DMSO control. It is important to note that although all compounds displayed some estrogenic activity on the target genes tested, the estrogenic response was not as robust as that of E2.

DISCUSSION

Numerous studies have investigated the relationship between PAHs, their hydroxylated metabolites, and potential interactions with the ERs, yet most have focused on ER α (reviewed by Santodonato, 1997). Hayakawa *et al.* (2007) reported estrogenic and antiestrogenic activity for multiple monohydroxylated derivatives of common PAHs in a yeast two-hybrid assay expressing ER α . Similar to our findings, they also reported that the parent PAH compounds lacked any estrogenic or antiestrogenic activity. Charles *et al.* (2000) also reported estrogenic activity for hydroxylated metabolites of the carcinogen benzo[a]pyrene (B[a]P) in MCF-7 cells, which primarily express ER α . Despite these previous findings, there have been relatively few studies comparing the effects of monohydroxylated PAHs on the differential activation and dimerization of ER α and ER β .

Our results, consistent with prior studies, indicate that hydroxylated PAHs are the active estrogenic species and can differentially activate either ER α or ER β . Although the compounds we tested exhibited no interaction with ER α , the interaction with ER β is novel and significant. Inhibition of luciferase signal by the ER antagonist ICI 182,780, as well as the lack of luciferase signal in the absence of Dox, demonstrates that the results of the reporter assay are ER β mediated. Competition with fluorescein-labeled estradiol indicates that these monohydroxylated PAH compounds directly bind to ER β at the same ligand-binding pocket as E2. Fertuck *et al.* (2001a) investigated different parental PAH and metabolite compounds, and they similarly reported that hydroxylated PAHs were able to compete with estrogen and bind ERs with a slight preference for ER β . Their data, consistent with our findings, suggest that hydroxylated PAHs may preferentially affect ER β signaling. Given ER β 's role in normal development and function in reproductive tissues as well as in the lungs, colon, prostate, and cardiovascular system, disruption of and interference with ER β signaling could have implications in normal development, as well as in cancers and malfunctions of these tissues.

In addition to the reporter assay and competitive binding data, the BRET and qPCR data confirm that 9-OH phenanthrene and 1-OH pyrene induce a biologically active ER β response in this



system. Given our data, 1-OH naphthalene may not necessarily induce ER β homodimers even at the high concentration tested (10 μ M). In support of these data, ligand-binding assays with 1-OH naphthalene demonstrate a relatively low binding affinity for ER β . Despite the negative BRET data, qPCR for endogenous ER β target genes suggest that 1-OH naphthalene is capable of inducing a slight biologically active ER β response for some ER β target genes although not to the same extent as E2. Collectively, the data obtained for 1-OH naphthalene demonstrate an important consideration of the *in vitro* assays used in this study: different assays have different sensitivities for detecting estrogenic activity and ER subtype selectivity. The ER β homodimerization BRET assay typically shows a 1.5- to 2-fold induction with E2 treatment because of high ligand-independent dimerization (Powell and Xu, 2008). In addition, the BRET ratios ultimately depend on the conformational changes within the receptor fusion proteins, which allow for efficient energy transfer, and different ligands will induce different conformational changes, thereby affecting the BRET ratio output. Despite the lower fold changes for the ER β homodimerization assay, BRET has been successfully used in a high-throughput manner to identify ER dimer selective ligands (Powell *et al.*, 2010) and, in this study, demonstrated a significant induction of ER β homodimerization by two other monohydroxylated PAHs, 1-OH pyrene and 9-OH phenanthrene.

Although each monohydroxylated PAH tested gave a similar pattern of results, the relative activity of each compound is quite different. Our data indicate that 1-OH naphthalene is the weakest ER β agonist among the tested metabolites, as demonstrated by low reporter gene output, a lack of saturation in the dose-response reporter assays, low induction of ER β dimerization, and a lower binding affinity for ER β . In contrast, 1-OH pyrene and 9-OH phenanthrene appear to be fairly efficient ER β agonists. Both ligands induced ERE-reporter gene activity similar to E2 and effectively displaced E2 from the ER β ligand-binding pocket. Both compounds also significantly elicited ER β homodimerization. 9-OH Phenanthrene generated data similar to 1-OH pyrene with the exception that it proved to be cytotoxic at concentrations greater than 10 μ M, resulting in difficulty to obtain accurate EC₅₀ values. Despite the cytotoxicity of 9-OH phenanthrene at high concentrations, treatment with lower concentrations of 9-OH phenanthrene (5 μ M) stimulated the regulation of endogenous ER β target genes in Hs578T-ER β Luc cells. These data suggest that some monohydroxylated PAHs can affect ER β -mediated signaling prior to inducing general cytotoxicity.

FIG. 6. Monohydroxylated PAHs can regulate ER β target genes similar to estradiol. Expression of ER β target genes (CC3, NR1P1, and JAG1) was determined by measuring relative mRNA levels using qPCR. RNA was collected following treatment with 0.1% DMSO, 10nM E2, 10 μ M 1-OH naphthalene, 5 μ M 9-OH phenanthrene, or 5 μ M 1-OH pyrene for 24-h and 48-h treatment with 50ng/ml Dox. Data are expressed as fold induction compared with DMSO control. Error bars represent SEM. * p < 0.05 compared with DMSO control.

Although our data did not indicate that any of the monohydroxylated PAHs tested had an effect on ER α , others have reported ER α estrogenic effects for these compounds. Hayakawa *et al.* (2007) reported that all three monohydroxylated PAHs exhibited little to no ER α estrogenic activity, but that 1-OH pyrene was able to compete with E2 for ER α binding. Additionally, Wiele *et al.* (2004) reported that 1-OH pyrene showed ER α estrogenic activity in colon extracts from a simulator of the human intestinal microbial ecosystem. Discrepancies across these studies may be due to the use of different assays and cell lines to assess the estrogenic activity.

Overall, these data suggest that common monohydroxylated PAHs can interact, positively or negatively, with ER signaling. We can conclude from our results and from other studies that hydroxylated PAHs are the active estrogenic species and can differentially bind ER α or ER β , likely in a cell- and tissue-specific manner. Few studies assessing the physiological serum concentrations of monohydroxylated PAHs have been published, although monohydroxylated PAHs may be used as urine biomarkers to assess exposure to PAHs (Elovaara *et al.*, 2006). It is therefore difficult to predict the concentrations of monohydroxylated PAHs that reach tissues such as the mammary gland, and the concentrations shown to be estrogenic in these studies may or may not be reached in the serum. Some estrogenic compounds in the diet, such as genistein found in soy products, can reach serum concentrations near the micromolar range (Cassidy *et al.*, 2006). Ultimately, the physiological exposure to monohydroxylated PAHs will be a function of both exposure and metabolic activity, which will greatly vary among individuals. These *in vitro* studies, however, demonstrate the potential for monohydroxylated PAHs to impact ER β -mediated signaling and provide a framework for assessing the impacts of other environmental chemicals on the dimerization and transcriptional activities of ER α and ER β .

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

FUNDING

National Institutes of Health (R01-CA125387 to W.X.); the National Institute of Environmental Health and Sciences (T32 ES007015 to E.S.); the Department of Defense Breast Cancer Research Program (Era of Hope Scholar Award to W.X. and predoctoral training grant to E.S.); the Greater Milwaukee Foundation (Shaw Scientist Award to W.X.).

ACKNOWLEDGMENTS

There are no conflicts of interest.

REFERENCES

- Anzenbacher, P., and Anzenbacherová, E. (2001). Cytochromes P450 and metabolism of xenobiotics. *Cell. Mol. Life Sci.* **58**, 737–747.
- Arcaro, K. F., O'Keefe, P. W., Yang, Y., Clayton, W., and Gierthy, J. F. (1999). Antiestrogenicity of environmental polycyclic aromatic hydrocarbons in human breast cancer cells. *Toxicology* **133**, 115–127.
- Baird, W. M., Hooven, L. A., and Mahadevan, B. (2005). Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environ. Mol. Mutagen.* **45**, 106–114.
- Bauer, E., Guo, Z., Ueng, Y. F., Bell, L. C., Zeldin, D., and Guengerich, F. P. (1995). Oxidation of benzo[a]pyrene by recombinant human cytochrome P450 enzymes. *Chem. Res. Toxicol.* **8**, 136–142.
- Cassidy, A., Brown, J. E., Hawdon, A., Faughnan, M. S., King, L. J., Millward, J., Zimmer-Nechemias, L., Wolfe, B., and Setchell, K. D. (2006). Factors affecting the bioavailability of soy isoflavones in humans after ingestion of physiologically relevant levels from different soy foods. *J. Nutr.* **136**, 45–51.
- Charles, G. D., Bartels, M. J., Zacharewski, T. R., Gollapudi, B. B., Freshour, N. L., and Carney, E. W. (2000). Activity of benzo[a]pyrene and its hydroxylated metabolites in an estrogen receptor- α reporter gene assay. *Toxicol. Sci.* **55**, 320–326.
- Cho, T. M., Rose, R. L., and Hodgson, E. (2006). In vitro metabolism of naphthalene by human liver microsomal cytochrome P450 enzymes. *Drug Metab. Dispos.* **34**, 176–183.
- Elovaara, E., Mikkola, J., Mäkelä, M., Paldanius, B., and Priha, E. (2006). Assessment of soil remediation workers' exposure to polycyclic aromatic hydrocarbons (PAH): Biomonitoring of naphthols, phenanthrols, and 1-hydroxypyrene in urine. *Toxicol. Lett.* **162**, 158–163.
- Fertuck, K. C., Kumar, S., Sikka, H. C., Matthews, J. B., and Zacharewski, T. R. (2001a). Interaction of PAH-related compounds with the α and β isoforms of the estrogen receptor. *Toxicol. Lett.* **121**, 167–177.
- Fertuck, K. C., Matthews, J. B., and Zacharewski, T. R. (2001b). Hydroxylated benzo[a]pyrene metabolites are responsible for in vitro estrogen receptor-mediated gene expression induced by benzo[a]pyrene, but do not elicit uterotropic effects in vivo. *Toxicol. Sci.* **59**, 231–240.
- Hartman, J., Lindberg, K., Morani, A., Inzunza, J., Ström, A., and Gustafsson, J. A. (2006). Estrogen receptor β inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res.* **66**, 11207–11213.
- Hayakawa, K., Onoda, Y., Tachikawa, C., Hosoi, S., Yoshida, M., Chung, S., Kizu, R., Toriba, A., Kameda, T., and Tang, N. (2007). Estrogenic/antiestrogenic activities of polycyclic aromatic hydrocarbons and their monohydroxylated derivatives by yeast two-hybrid assay. *J. Health Sci.* **53**, 562–570.
- Kim, J. H., Stansbury, K. H., Walker, N. J., Trush, M. A., Strickland, P. T., and Sutter, T. R. (1998). Metabolism of benzo[a]pyrene and benzo[a]pyrene-7,8-diol by human cytochrome P450 1B1. *Carcinogenesis* **19**, 1847–1853.
- Koterba, K. L., and Rowan, B. G. (2006). Measuring ligand-dependent and ligand-independent interactions between nuclear receptors and associated proteins using Bioluminescence Resonance Energy Transfer (BRET). *Nucl. Recept. Signal.* **4**, e021.
- Paruthiyil, S., Parmar, H., Kerekatte, V., Cunha, G. R., Firestone, G. L., and Leitman, D. C. (2004). Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res.* **64**, 423–428.
- Pike, A. C., Brzozowski, A. M., Hubbard, R. E., Bonn, T., Thorsell, A. G., Engström, O., Ljunggren, J., Gustafsson, J. A., and Carlquist, M. (1999). Structure of the ligand-binding domain of oestrogen receptor β in the presence of a partial agonist and a full antagonist. *EMBO J.* **18**, 4608–4618.
- Powell, E., Huang, S. X., Xu, Y., Rajski, S. R., Wang, Y., Peters, N., Guo, S., Xu, H. E., Hoffmann, F. M., Shen, B., *et al.* (2010). Identification and

- characterization of a novel estrogenic ligand actinopolymorphol A. *Biochem. Pharmacol.* **80**, 1221–1229.
- Powell, E., and Xu, W. (2008). Intermolecular interactions identify ligand-selective activity of estrogen receptor alpha/beta dimers. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 19012–19017.
- Rossbach, B., Preuss, R., Letzel, S., Drexler, H., and Angerer, J. (2007). Biological monitoring of occupational exposure to polycyclic aromatic hydrocarbons (PAH) by determination of monohydroxylated metabolites of phenanthrene and pyrene in urine. *Int. Arch. Occup. Environ. Health* **81**, 221–229.
- Santodonato, J. (1997). Review of the estrogenic and antiestrogenic activity of polycyclic aromatic hydrocarbons: Relationship to carcinogenicity. *Chemosphere* **34**, 835–848.
- Secreto, F. J., Monroe, D. G., Dutta, S., Ingle, J. N., and Spelsberg, T. C. (2007). Estrogen receptor alpha/beta isoforms, but not beta α , modulate unique patterns of gene expression and cell proliferation in Hs578T cells. *J. Cell. Biochem.* **101**, 1125–1147.
- Shanle, E. K., Hawse, J. R., and Xu, W. (2011). Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. *Biochem. Pharmacol.* **82**, 1940–1949.
- Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989). The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**, 477–487.
- Trecek, O., Latrich, C., Springwald, A., and Ortmann, O. (2010). Estrogen receptor beta exerts growth-inhibitory effects on human mammary epithelial cells. *Breast Cancer Res. Treat.* **120**, 557–565.
- Tremblay, A., Tremblay, G. B., Labrie, F., and Giguère, V. (1999). Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Mol. Cell* **3**, 513–519.
- Wiele, T. V. D., Vanhaecke, L., Boeckaert, C., Peru, K., Headley, J., Verstraete, W., and Siciliano, S. (2005). Human colon microbiota transform polycyclic aromatic hydrocarbons to estrogenic metabolites. *Environ. Health Perspect.* **113**, 6–10.

Research Resource: Global Identification of Estrogen Receptor β Target Genes in Triple Negative Breast Cancer Cells

Erin K. Shanle, Zibo Zhao, John Hawse, Kari Wisinski, Sunduz Keles, Ming Yuan, and Wei Xu

Molecular and Environmental Toxicology Center (E.K.S., W.X.) and McArdle Laboratory for Cancer Research (E.K.S., Z.Z., W.X.), University of Wisconsin, Madison, Wisconsin; Department of Biochemistry and Molecular Biology (J.H.), Mayo Clinic, Rochester, Minnesota; University of Wisconsin Carbone Cancer Center (K.W.), Madison, Wisconsin; Departments of Statistics and Biostatistics and Medical Informatics (S.K.), University of Wisconsin, Madison, Wisconsin; and Morgridge Institute for Research (M.Y.) and Department of Statistics, University of Wisconsin, Madison, Wisconsin

Breast cancers that are negative for estrogen receptor α (ER α), progesterone receptor, and human epidermal growth factor receptor 2 are known as triple-negative breast cancers (TNBC). TNBCs are associated with an overall poor prognosis because they lack expression of therapeutic targets like ER α and are biologically more aggressive. A second estrogen receptor, ER β , has been found to be expressed in 50% to 90% of ER α -negative breast cancers, and ER β expression in TNBCs has been shown to correlate with improved disease-free survival and good prognosis. To elucidate the role of ER β in regulating gene expression and cell proliferation in TNBC cells, the TNBC cell line MDA-MB-468 was engineered with inducible expression of full-length ER β . In culture, ER β expression inhibited cell growth by inducing a G1 cell cycle arrest, which was further enhanced by 17 β -estradiol treatment. In xenografts, ER β expression also inhibited tumor formation and growth, and 17 β -estradiol treatment resulted in rapid tumor regression. Furthermore, genomic RNA sequencing identified both ligand-dependent and -independent ER β target genes, some of which were also regulated by ER β in other TNBC cell lines and correlated with ER β expression in a cohort of TNBCs from the Cancer Genome Atlas Network. ER β target genes were enriched in genes that regulate cell death and survival, cell movement, cell development, and growth and proliferation, as well as genes involved in the Wnt/ β -catenin and the G1/S cell cycle phase checkpoint pathways. In addition to confirming the anti-proliferative effects of ER β in TNBC cells, these data provide a comprehensive resource of ER β target genes and suggest that ER β may be targeted with ligands that can stimulate its growth inhibitory effects. (*Molecular Endocrinology* 27: 1762–1775, 2013)

Estrogen signaling is primarily mediated by two estrogen receptors (ERs): ER α and ER β . ER α is expressed in approximately 70% of breast cancers, and many of these cancers respond to endocrine therapies that block the proliferative action of ER α . However, approximately 15% to 20% of all breast cancers lack expression of ER α , its target gene progesterone receptor (PR), and human

epidermal growth factor receptor 2 (HER2) and are clinically defined as triple-negative breast cancers (TNBCs). Full-length ER β protein has been detected in 50% to 90% of ER α -negative breast cancers (1–4), and ER β expression has been shown to correlate with improved disease-free survival and good prognosis in TNBC (2). Unlike ER α , for which PR expression is indicative of receptor

ISSN Print 0888-8809 ISSN Online 1944-9917

Printed in U.S.A.

Copyright © 2013 by The Endocrine Society

Received June 6, 2013. Accepted August 19, 2013.

First Published Online August 26, 2013

Abbreviations: Dox, doxycycline; E2, 17 β -estradiol; ER α , estrogen receptor α ; ER β , estrogen receptor β ; FBS, fetal bovine serum; GFP, green fluorescent protein; IPA, Ingenuity pathway analysis; HER2, human epidermal growth factor receptor 2; 4-OH Tam, 4-hydroxytamoxifen; PR, progesterone receptor; RSEM, RNA sequencing expectation maximization; RNA-seq, RNA sequencing; SFS, charcoal stripped FBS; TCGA, the Cancer Genome Atlas; Tet, tetracycline; TNBC, triple-negative breast cancers.

expression and function, a target gene or gene set indicating ER β functionality has yet to be identified.

Like ER α , ER β is a nuclear receptor that regulates target gene expression in estrogen responsive tissues, such as the mammary gland. Multiple isoforms of ER β may be expressed in the mammary gland, but the full-length receptor is the only isoform able to bind ligand with high affinity and regulate target gene expression (5, 6). Several studies have assessed the effects of full-length ER β expression on the growth of ER α -positive breast cancer cells (7–11). The results of these studies demonstrate that ER β expression inhibits the proliferative response mediated by ER α . Fewer reports have assessed the growth effects of ER β expression in breast cancer cells that lack ER α , but ER β expression in ER α -negative breast cancer cells has been shown to inhibit growth in ligand-independent and -dependent manners (12–15). This leads to the hypothesis that some ER α -negative breast cancers, including TNBCs, may benefit from therapies that target ER β (16).

In regard to gene expression, even fewer studies have aimed to identify ER β target genes in the absence of ER α . Microarray analyses comparing ER α and ER β target genes in U2OS osteosarcoma cells (17) and Hs578T breast cancer cells (15) demonstrated that the two receptors have both overlapping and distinct target genes. However, only ligand-dependent ER β target genes have been identified in ER α -negative breast cancer cells, and a comprehensive assessment of both ligand-independent and -dependent ER β target genes in TNBC cells has not yet been completed.

In an effort to identify ER β target genes globally in TNBC cells and assess the growth inhibition of ER β expression in vitro and in vivo, we generated a TNBC cell line with inducible expression of full-length ER β . We confirmed ER β -mediated growth inhibition in vitro and in vivo and identified ER β target genes using RNA sequencing. We further show that some of the ER β target genes are associated with ER β expression in a cohort of TNBCs. These data demonstrate the growth inhibitory properties of ER β and provide a global view of ligand-independent and -dependent ER β target genes in the absence of ER α expression. This study also provides a foundation to identify target genes further that may indicate ER β functionality in TNBCs.

Materials and Methods

Cell culture and reagents

Cell culture media were obtained from Invitrogen. MDA-MB-468 cells were cultured in DMEM + 10% fetal bovine serum (FBS; Invitrogen), and BT549 and HCC1143 cells were

cultured in RPMI 1640 + 10% FBS. HCC1143 breast cancer cells were obtained from the Leibniz Institute DSMZ, German Institute for Microorganisms and Cell Culture. All cells were cultured at 37°C and 5% CO₂. MDA-MB-468-ER β inducible cells were cultured in DMEM + 10% Tet-system approved FBS (Clontech Mountain View), 500 mg/L Zeocin, and 5 mg/L Blasticidin S (Research Products International). Before all experiments, MDA-MB-468-ER β cells were cultured in phenol red free DMEM + 10% charcoal stripped FBS (SFS) for at least 3 days to remove residual estrogens. Doxycycline (Dox) was obtained from Clontech. The Flag and β -actin antibodies were obtained from Sigma-Aldrich, and the Hsp90 antibody was obtained from Santa Cruz Biotechnology. ICI 182,780 and ERB-041 were purchased from Tocris. All other reagents were purchased from Sigma Aldrich unless otherwise noted.

Generation of MDA-MB-468-ER β inducible cells

MDA-MB-468-ER β inducible cells were created as previously described using the TRex system (Invitrogen) (15). Briefly, MDA-MB-468 cells were transfected with pcDNA6/TR, and a stable clone was selected after selection with 5 μ g/mL Blasticidin S. The MDA-MB-468-Tet cells were then transfected with pcDNA4/TO-ER β , which encodes the 530 amino acids, full-length ER β isoform, and selected with 500 μ g/mL Zeocin. Stable clones were selected and screened for inducible expression of ER β using Western blotting with Flag antibody.

Ligand binding assays and Western blots

Ligand binding assays were performed as previously described with slight modification (18). Briefly, 2×10^5 MDA-MB-468-ER β cells were seeded in a 24-well plate and treated with vehicle or 50 ng/mL Dox. After 48 hours, cells were incubated with 20 nM radiolabeled [³H]-E2 (89.2 Ci/mmol specific activity; Perkin Elmer) in the presence or absence of 450 nM diethylstilbestrol cold competitor. After a 2-hour incubation, cells were washed and lysed and the retained radioactivity was determined using a liquid scintillation counter.

To assess ER β expression using Western blots, MDA-MB-468-ER β cells were treated with the corresponding Dox concentrations for the given amount of time. Cells were then washed in PBS and lysed as previously described (18). After separation by SDS-PAGE and transfer to a nitrocellulose membrane, the membrane was incubated with the corresponding antibody and visualized by enhanced chemiluminescence on a Chemidoc XRS system (BioRad) or by fluorescence detection on a Licor Odyssey gel reader (Licor Biosciences) as previously described (18).

Phalloidin staining

MDA-MB-468 ER β #18 cells were maintained in DMEM supplemented with 10% stripped FBS for 3 days. The cells were then cultured in DMEM + 5% SFS with or without Dox to induce ER β expression. After 24 hours, the cells were treated with or without 10 nM E2. Forty-eight hours before immunostaining, the cells were seeded onto coverslips in a 6-well plate. After 5 days of treatment, the cells were fixed in 4% formaldehyde for 15 minutes, and the coverslips were washed three times in PBS. Alexa Fluor 555 Phalloidin (Cell Signaling Technology) was diluted 1:20 (6.6 μ M stock concentration in methanol) in PBS and added to the cells. After 15 minutes incubation at room temperature, coverslips were washed once with PBS. The cov-

erslips were mounted in ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen). Fluorescence was detected using a Leica DM5000B microscope (Buffalo Grove) with the appropriate wavelengths.

Cell proliferation and cell cycle assays

For cell proliferation assays, 1×10^5 MDA-MB-468-ER β cells were seeded in triplicate onto 6-well plates in DMEM + 5% SFS. After 24 hours, cells were treated with vehicle or 50 ng/mL Dox for 24 hours. Cells were then treated with the corresponding ligands or 0.1% DMSO for the given amount of time. Media were refreshed every 48 hours. Cells were trypsinized and counted after trypan blue exclusion using an automated cell counter (BioRad) according to the manufacturer's protocol.

To assess the cell cycle distribution, 3×10^5 MDA-MB-468-ER β cells were seeded in triplicate onto 6-cm plates in DMEM + 5% SFS. After 24 hours, the cells were treated with vehicle or 50 ng/mL Dox for 24 hours followed by treatment with 0.15% DMSO or the corresponding ligands for 72 hours. Cells were collected by trypsinization, fixed in cold 95% ethanol, and washed in PBS. The fixed cells were then resuspended in propidium iodide staining solution (200 μ g/mL RNase A, 50 μ g/mL propidium iodide, 0.1% [v/v] Triton X-100 in PBS + 1% BSA) and incubated overnight at 4°C. Samples were analyzed by flow cytometry at the University of Wisconsin Flow Cytometry Laboratory.

Xenograft experiments

All animal work was performed in accordance with protocols approved by the Animal Care and Use Committee of the University of Wisconsin-Madison. 468-ER β #18 cells were infected with retrovirus generated from a pLNCX-FLuc (firefly luciferase) vector. Cells were then cultured in regular media containing 400 μ g/mL G418 (Invitrogen) for at least eight passages to generate luciferase-labeled 468-ER β #18 cells. To assess the effects of ER β expression on tumorigenicity, 2×10^6 luciferase-labeled 468-ER β #18 cells were injected bilaterally into the inguinal mammary fat pads of 5- to 6-week-old ovariectomized athymic nude-Foxn1nu mice ($n = 4$ per group). Mice were given control diets or Dox-containing diets (2000 ppm) obtained from Harlan Laboratories immediately after the injections. To assess tumor regression in response to E2, 2×10^6 luciferase-labeled 468-ER β #18 cells were injected as described above, and the tumors were allowed to form in the absence of Dox for 10 weeks. On day 0, a 60-day release 0.05 mg E2 pellet (Innovative Research of America) was implanted into each mouse. Mice ($n = 3$ per group) were then separated randomly and given a control or Dox-containing diet. Luciferase-based noninvasive bioluminescent imaging and analysis were performed as previously described with an IVIS Imaging System (Caliper Life Sciences) (19). Briefly, mice were anesthetized and injected ip with 2 mg D-luciferin (10 mg/mL in PBS) (Gold Biotechnology). Imaging was completed between 15 and 20 minutes after injection. For bioluminescence plots, total photon flux was calculated for each mouse by using a circular region of interest.

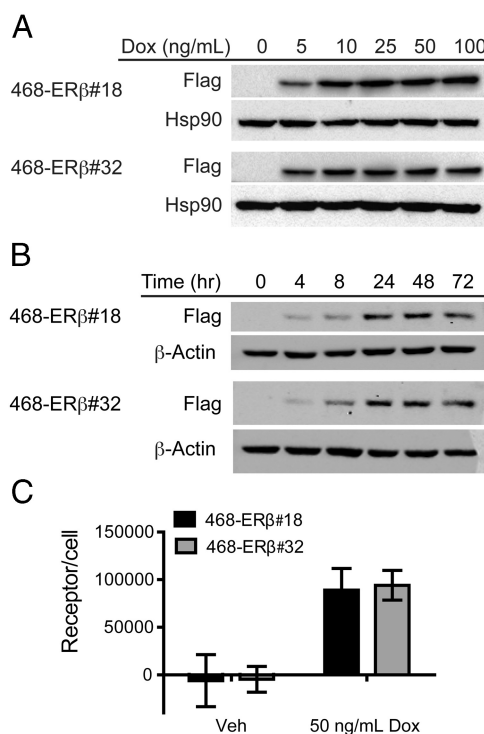


Figure 1. MDA-MB-468-ER β cells express ER β after Dox treatment. Dox-dependent expression of ER β was initially characterized in two MDA-MB-468-ER β clones (468-ER β #18 and 468-ER β #32). (A) A Western blot using Flag antibody shows that both clones express detectable levels of ER β after treatment with as little as 5 ng/mL Dox for 48 hours. Further in vitro studies were performed with 50 ng/mL Dox. (B) A Western blot using Flag antibody shows that treatment with 50 ng/mL Dox for as little as 4 hours induces expression of ER β . (C) Ligand binding assays performed with radiolabeled E2 demonstrate that both clones express similar levels of ER β corresponding to approximately 90 000 receptors/cell after 24 hours of Dox treatment. The data represent the mean and SD.

RNA sequencing (RNA-seq) of ER β target genes and pathway analysis

To identify ER β target genes globally, 468-ER β #18 cells were treated with vehicle or 50 ng/mL Dox for 48 hours followed by treatment with 0.1% DMSO or 10 nM E2 for 4 hours. Total RNA was extracted using a RNeasy Plus Kit according to manufacturer protocol (Qiagen), and three independent experiments were performed. The total RNA was submitted to the University of Wisconsin Biotechnology Center for RNA quality analysis, sequencing library generation, and sequencing. An Illumina HiSeq 2000 was used to generate 100-bp single-end reads. Reads were trimmed for quality (15 bp from the 5' end and 2 bp from the 3' end) and aligned to the University of California, Santa Cruz build hg19 genome using RNA sequencing expectation maximization (RSEM). It has now been well recognized that discarding RNA-seq reads that align to multiple genes introduces bias in expression estimates of gene families and genes containing or inside of repetitive elements (20–23). RSEM implements a principled and biologically motivated probabilistic model for this problem (20, 24). It is based on a generative model of the RNA-seq protocol that takes into account aspects such as sequencing error, fragment length distributions, and nonuniformities in the distribution of read loca-

tions along transcripts. The RSEM alignment results are presented in Supplemental Table 2, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>. For the identification of differentially expressed genes, the fractional counts were rounded to the nearest integer, and DESeq (25) was used to assess differentially expressed genes at a 1.5-fold change cutoff and a false discovery rate <0.01 . The filtered gene lists for each comparison are provided in Supplemental Materials and Methods. The raw data will also be submitted to the Gene Expression Omnibus. Pathway enrichments and functional analyses of the gene lists were determined using ingenuity pathway analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). The DESeq and IPA results for all com-

parisons are available in the Supplemental Materials and Methods.

Quantitative PCR

468-ER β #18 cells were treated with DMSO, 10 nM E2, or 100 nM ERB-041 as described for the initial RNA-seq experiment. For validation in HCC1143 cells, the cells were infected with retrovirus generated from pLNCX-GFP or pLPCX-ER β vectors. Twenty-four hours after infection, the media were replaced with phenol red free RPMI 1640 + 5% SFS. After an additional 24 hours, cells were treated with 0.1% DMSO or 10 nM E2 for 4 hours. Total RNA was extracted as described above

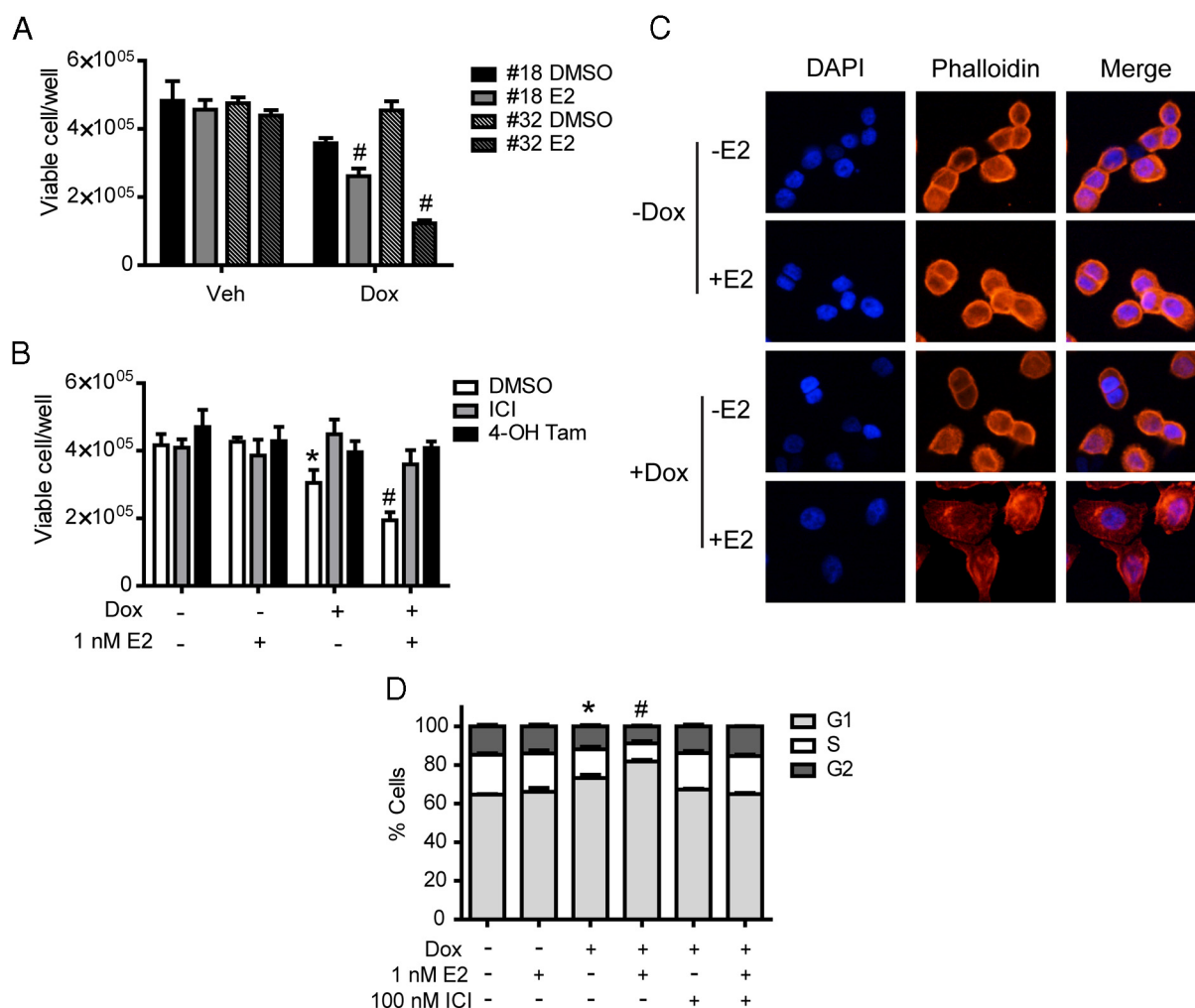


Figure 2. ER β expression and E2 treatment inhibit the growth of MDA-MB-468-ER β cells. (A) Two MDA-MB-468-ER β clones (#18 and #32) were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or 1 nM E2. The total number of viable cells was determined using trypan blue exclusion after the given number of days. *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. (B) Treatment with ER antagonists rescue the growth inhibition mediated by ER β . 468-ER β #18 cells were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 in the presence or absence of 100 nM ICI or 4-OH Tam. The total number of viable cells was determined by trypan blue exclusion after 5 days. *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. (C) ER β expression and E2 treatment alter the morphology of the 468-ER β #18 cells. Cells were treated with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 for 5 days and subsequently stained with Alexa Fluor 555 Phalloidin (red), which stains actin filaments to highlight the cytoplasm, and 4',6-diamidino-2-phenylindole (blue) for nuclear staining. (D) ER β expression and E2 treatment induce a G1 cell cycle arrest. 468-ER β #18 cells were treated in triplicate with vehicle or Dox for 24 hours followed by treatment with DMSO or E2 in the presence or absence of 100 nM ICI for 72 hours. The proportion of cells in each phase of the cell cycle was determined using flow cytometry as described in the *Materials and Methods*. For the proportion of cells in G1; *, $P < .01$ compared to DMSO control; #, $P < .01$ compared to DMSO+Dox control. The data represent the mean and SD.

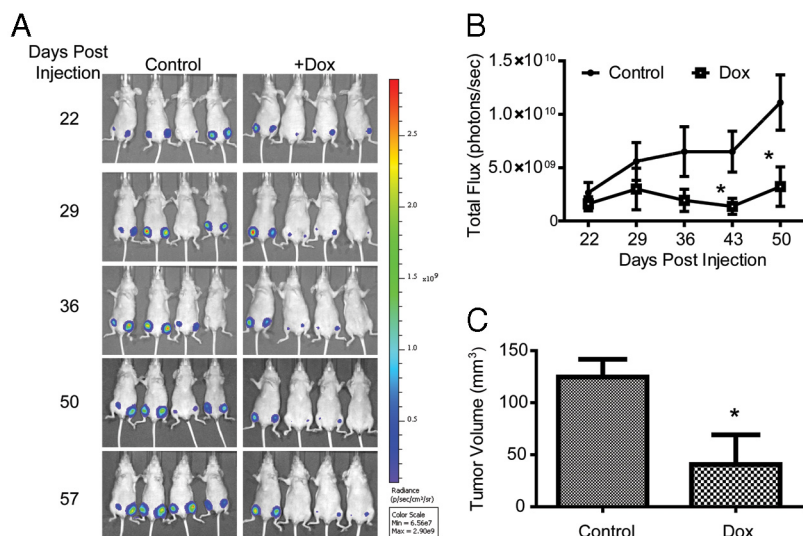


Figure 3. ER β expression inhibits tumor growth in xenografts. (A) 468-ER β #18 cells were labeled with luciferase and injected into the mammary fat pads of nude mice ($n = 4$ per group). Mice were then provided a control or Dox-containing diet. The tumor growth was monitored using bioluminescence imaging as described in the *Materials and Methods*. (B) Quantitative plot of bioluminescence for each group over time. (C) Final tumor volumes for each group. The data represent the mean and SEM of the tumors from each group. *, $P < .05$ compared to control.

and reverse-transcribed using Superscript II RT according to the manufacturer's protocol (Invitrogen). Quantitative PCR was performed using TaqMan Prime Time custom designed assays (IDT), FastStart Universal Probe Master Mix (Roche Scientific), and a CFX96 instrument (BioRad). Primer and probe sequences are shown in Supplemental Table 1. Data were analyzed using the $\Delta\Delta C_q$ method calculated by CFX Manager Software (BioRad). Student t tests were performed with GraphPad Prism Software (Version 5.04; GraphPad Software Inc).

Correlation analysis of ER β target genes and ER β expression in human tumor samples

To compare the RNA sequencing results further with previous studies of TNBC, the Agilent mRNA expression microarray data from the Cancer Genome Atlas (TCGA) database, among which $n = 43$ samples were identified as TNBC, was examined to assess any correlations between the expression of ER β and its target genes (26). Using all of the genes identified in the comparison of 468-ER β #18 cells treated with E2 versus E2+Dox (comparison IV, Figure 5A) with false discovery rate <0.05 , a total of 2472 genes were present in both the microarray data and our gene list. The correlation coefficient was calculated for the expression of each of the genes and that of ER β based on the microarray data. To assess the strength of the correlation, the P values were calculated for a two-sided alternative that these correlation coefficients are significantly different from zero (meaning no correlation). To account for multiple testing, these P values were then converted to the so-called q values (27, 28). For those genes with $q < .05$ for the correlation and a fold change >1.5 in the RNA sequencing results, the correlation coefficient determined for the array data was graphed against the fold change observed for the gene in the 468-ER β #18 cells treated with E2 versus E2+Dox.

Results

Inducible expression of ER β and E2 treatment induces a G1 cell cycle arrest and inhibits the growth of MDA-MB-468 cells in vitro and in vivo

To assess the growth and gene expression effects of ER β expression and activation in TNBC cells, MDA-MB-468 cells were engineered with inducible expression of full-length ER β using the TRex system from Invitrogen. After creating MDA-MB-468-Tet cells, which express the tetracycline (Tet) repressor, these cells were transfected with a construct encoding Tet-operated full-length Flag-tagged ER β . Two MDA-MB-468-ER β clones with inducible ER β expression were selected for further characterization (486-ER β #18 and 468-ER β #32). First, ER β expression

was assessed in response to increasing Dox concentrations (Figure 1A). As little as 5 ng/mL Dox treatment for 48 hours induced expression of the receptor, and subsequent in vitro experiments were performed with 50 ng/mL Dox. Next, a time course was established for the Dox-inducible expression of ER β (Figure 1B). ER β was maximally expressed after 24 hours, and this expression level was sustained after 48 hour and 72 hour treatments. Finally, the expression level was quantified using radio-labeled ligand binding assays (Figure 1C). After Dox treatment, both clones expressed approximately 90 000 receptors per cell, which is comparable to MCF7 breast cancer cells that express ~ 150 000 ER α molecules per cell (18).

After confirming Dox-inducible expression of ER β in 486-ER β #18 and 468-ER β #32 cells, we assessed the growth effects of ER β expression and activation by 17 β -estradiol (E2) (Figure 2A). The number of viable cells was significantly inhibited by ER β expression and E2 treatment for both clones. 468-ER β #18 showed slight growth inhibition when ER β was expressed in the absence of ligand. Dox and E2 treatment had no effect on the growth of MDA-MB-468-Tet cells (Supplemental Figure 1). To confirm that the growth inhibitory effects observed in MDA-MB-468-ER β cells were mediated by ER β , 468-ER β #18 cells were treated with vehicle or Dox and DMSO or E2 in the presence or absence of the antagonist ICI 182,780 (ICI) or the selective estrogen receptor modulator 4-hydroxytamoxifen (4-OH Tam) (Figure 2B).

Dox treatment alone significantly inhibited the growth of 468-ER β #18 cells, and this effect was rescued by ICI or 4-OH Tam co-treatment. Treatment with E2 further inhibited the growth of the cells, and this inhibition was also rescued by ICI or 4-OH Tam co-treatment. The ER β -selective ligand, ERB-041 (29), also inhibited cell growth similar to E2 (Supplemental Figure 2). Interestingly, the morphology of the cells was altered after 5 days of treatment with Dox and E2 (Figure 2C), suggesting that the cells may have undergone some type of cell cycle arrest. Indeed, an analysis of the cell cycle phase distribution by flow cytometry revealed an increase in the proportion of cells in the G1 phase after Dox treatment alone, which was further increased by E2 treatment (Figure 2D). ER β expression and ligand treatment did not induce detectable levels of apoptosis (data not shown).

Next, we aimed to assess the effects of ER β expression and activation on tumor growth of MDA-MB-468 cells. First, soft agar colony formation assays revealed that ER β expression alone completely blocked the formation of colonies (Supplemental Figure 3). Xenograft experiments were then performed using 468-ER β #18 cells retrovirally labeled with luciferase to monitor tumor growth by bioluminescence imaging (Figure 3). After injecting cells into the mammary fat pads, the mice were given a control diet or a Dox-containing diet, and the tumor size was monitored using bioluminescence imaging (Figure 3A). In the presence of Dox, the growth of the tumors was reduced and one mouse did not develop tumors (Figure 3, A and B). The final tumor volume was significantly smaller when ER β was expressed (Figure 3C), and Dox treatment had no effect on the growth of control MDA-MB-468-Tet tumors (Supplemental Figure 4). To assess the effects of ER β expression and activation on tumor growth, luciferase-labeled 468-ER β #18 cells were allowed to form tumors for 10 weeks. Mice were then implanted with a slow release E2 pellet and given a control or Dox-containing diet. The tumors rapidly regressed only when ER β was expressed (Figure 4). These data demonstrate the growth inhibitory effects of ER β expression and activation in TNBC cells in vivo.

ER β target gene identification using RNA-seq

The MDA-MB-468-ER β inducible cells are useful for identifying the ligand-independent and ligand-dependent target genes of ER β in the absence of ER α . To identify direct ER β target genes, 468-ER β #18 cells were treated with or without Dox followed by treatment with E2 for 4 hours. RNA-seq was subsequently performed as described in the *Materials and Methods*. Reads were aligned using RSEM (20), and the results of the alignment are shown in Supplemental Table 2. Differentially expressed

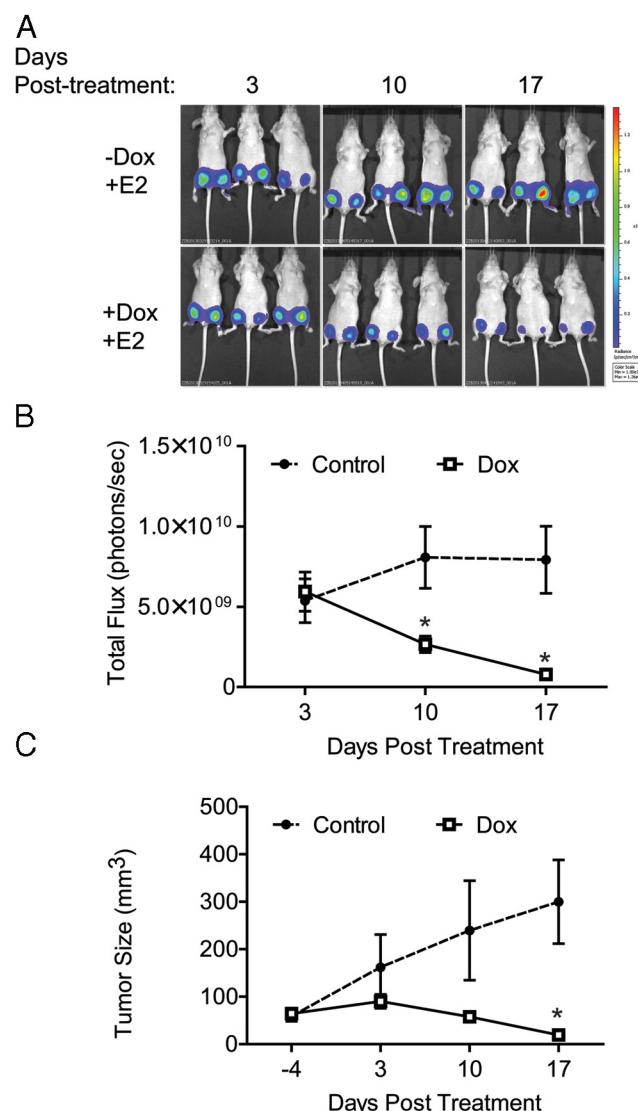


Figure 4. Activation of ER β in xenografts causes tumor regression. (A) 468-ER β #18 cells were labeled with luciferase and injected into the mammary fat pads of nude mice. After allowing tumors to form for 10 weeks, slow release E2 pellets were implanted into each mouse. Mice were separated into two groups ($n = 3$ per group) and provided a control or Dox-containing diet. The tumor growth was monitored using bioluminescence imaging and caliper measurements as described in the *Materials and Methods*. (B) Quantitative plot of bioluminescence for each group over time. The data represent the mean and SEM of the tumors from each group. (C) Quantitative plot of tumor size as determined by caliper measurements for each group over time. The data represent the mean and SEM of the tumors from each group. *, $P < .05$ compared to control.

genes were determined for the four conditions by DESeq (25) (Figure 5A, 1.5-fold change cutoff). No genes were differentially expressed after E2 treatment in the absence of ER β (DMSO vs E2, comparison I), confirming that MDA-MB-468 cells are not estrogen responsive. Only 109 genes were differentially expressed after Dox treatment (DMSO vs DMSO+ER β , comparison II), whereas 481 genes were differentially expressed after E2 treatment

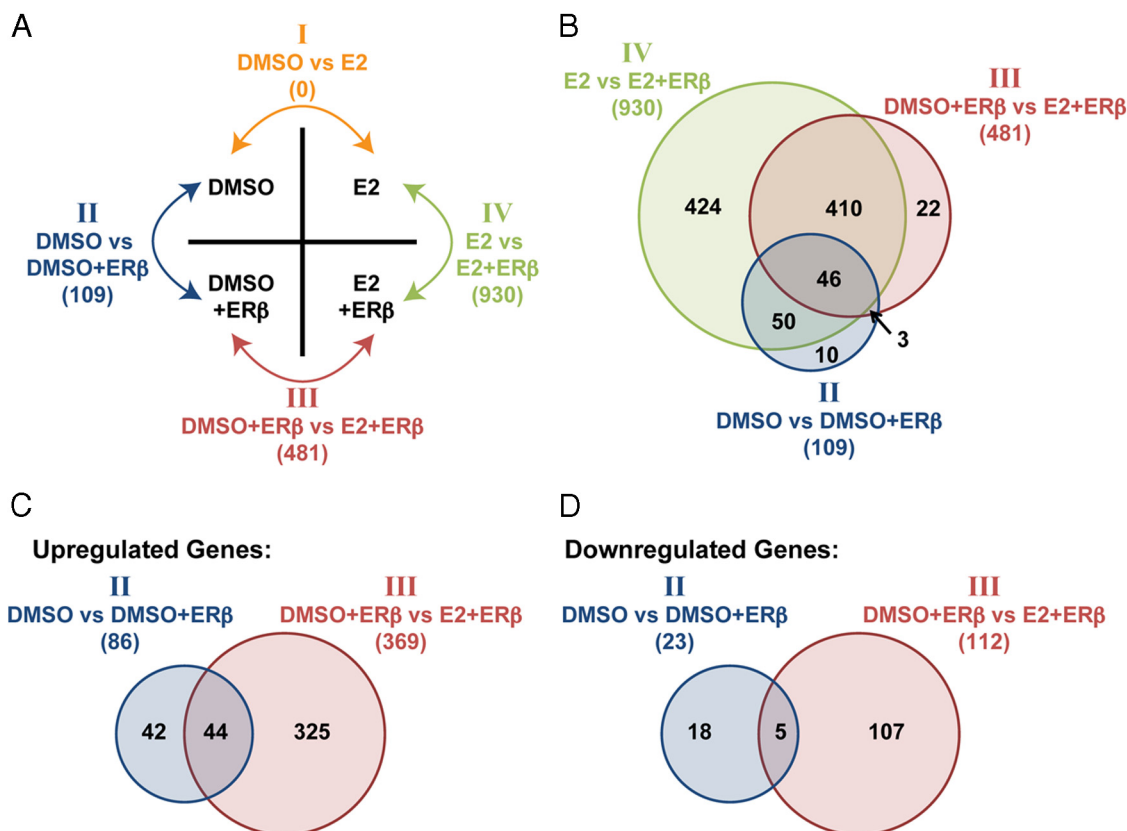


Figure 5. Global identification of ER β target genes using RNA-seq. 468-ER β #18 cells were treated with vehicle or Dox for 48 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Total RNA was extracted and sequenced as described in the *Materials and Methods*. Differentially expressed genes were identified using DESeq with a 1.5-fold change cutoff and a false discovery rate <0.01 . (A) A matrix describing the four contrasts performed to identify differentially expressed genes. No genes were differentially expressed in the DMSO vs E2 group (I), 109 were differentially expressed in the comparison between DMSO vs DMSO+ER β (II), 481 genes were differentially expressed between DMSO+ER β vs E2+ER β (III), and 930 genes were differentially expressed between E2 vs E2+ER β (IV). (B) A Venn diagram showing the overlap among genes identified in the three groups. (C) A Venn diagram showing the overlap between the up-regulated genes identified in DMSO vs DMSO+ER β and DMSO+ER β vs E2+ER β . Most ER β target genes were up-regulated, and approximately half of the DMSO vs DMSO+ER β genes were identified in DMSO+ER β vs E2+ER β . (D) A Venn diagram showing the overlap between the down-regulated genes identified in DMSO vs DMSO+ER β and DMSO+ER β vs E2+ER β . Fewer ER β target genes were down-regulated, and only five genes were identified in both groups.

in the presence of ER β (DMSO+ER β vs E2+ER β , comparison III), suggesting that most ER β target genes are ligand-dependent in these cells. The top genes regulated in comparisons II and III are shown in Tables 1 and 2, respectively. A comparison of E2 treatment with or without Dox (E2 vs E2+ER β , comparison IV) revealed 930 differentially expressed genes, potentially capturing the total change in gene expression in response to both ER β expression and activation by E2. The top genes in this comparison are shown in Table 3.

Most of the genes in comparisons II and III were captured in comparison IV (Figure 5B). In addition, most genes identified in comparisons II and III were up-regulated, and about half of the potentially ligand-independent genes (comparison II) were also regulated in a ligand-dependent manner (Figure 5C). Fewer genes were found to be down-regulated (Figure 5D). Several genes were validated by quantitative PCR in a new set of biological replicates of 468-ER β #18 cells treated with vehicle or

Dox followed by DMSO or E2 for 4 hours. Two ligand-independent genes were confirmed (*CTGF* and *C3*; Figure 6A) and three ligand-dependent genes were confirmed (*SDC1*, *CDH1*, and *DKK1*; Figure 6B). Four genes regulated in both a ligand-dependent and a ligand-independent manner were also validated (*WNT4*, *THRSF*, *CABLES1*, and *S100A7*; Figure 6C). Importantly, the expression of several ER β target genes was assessed in MDA-MB-468-Tet cells treated with vehicle or Dox, and none of the genes were regulated by Dox in the absence of ER β expression (Supplemental Figure 5). In addition, the ER β -selective ligand ERB-041 was found to regulate ER β target genes similar to E2 (Figure 7, A and B).

ER β target genes are enriched in pathways regulating cell proliferation, differentiation, and cell cycle

After globally identifying the ER β target genes in ER α -negative TNBC cells, we conducted a pathway analysis of

Table 1. Most Highly Up-regulated and Down-regulated Genes in the DMSO vs DMSO+ER β Comparison

Up-regulated Genes		Down-regulated Genes	
Gene Symbol	Fold Change	Gene Symbol	Fold Change
<i>ESR2</i>	92.93	<i>TMEM189-UBE2V1</i>	0.25
<i>FGF19</i>	44.42	<i>ACER1</i>	0.26
<i>PDZK1</i>	13.76	<i>NOV</i>	0.32
<i>SCGB2A2</i>	10.70	<i>ADAMTS5</i>	0.41
<i>THRSP</i>	9.25	<i>CTGF</i>	0.43
<i>EPHA8</i>	8.11	<i>NDRG1</i>	0.49
<i>RBP3</i>	7.55	<i>KDR</i>	0.52
<i>CA12</i>	6.28	<i>KRT6B</i>	0.54
<i>CYP2A13</i>	5.60	<i>NTRK2</i>	0.55
<i>MAPK4</i>	5.04	<i>KRT14</i>	0.57
<i>CD34</i>	4.77	<i>IGFBP3</i>	0.57
<i>KCNK15</i>	4.73	<i>CLIC5</i>	0.58
<i>CYP2B7P1</i>	4.10	<i>MAOA</i>	0.59
<i>C3</i>	3.69	<i>FOXQ1</i>	0.59
<i>ST8SIA6</i>	3.57	<i>SYTL4</i>	0.59
<i>S100A7A</i>	3.49	<i>KRT16</i>	0.61
<i>MGAT3</i>	3.42	<i>CASP14</i>	0.61
<i>S100A7</i>	3.39	<i>PPP1R3C</i>	0.62
<i>ANPEP</i>	3.31	<i>SMOC1</i>	0.63
<i>TMEM229B</i>	3.04	<i>KRT17</i>	0.63

the total ER β target genes identified in comparison IV (Figure 5A) using IPA. These ER β target genes were enriched in several molecular and cellular functions, including cell movement, cell death and necrosis, and cell dif-

Table 2. Most Highly Up-regulated and Down-regulated Genes in the DMSO+ER β vs E2+ER β Comparison

Up-regulated Genes		Down-regulated Genes	
Gene Symbol	Fold Change	Gene Symbol	Fold Change
<i>EGR3</i>	43.43	<i>ADAMTS5</i>	0.13
<i>OTOF</i>	34.18	<i>CHRM1</i>	0.25
<i>SHISA2</i>	26.24	<i>ADAMTS1</i>	0.27
<i>ASB2</i>	18.22	<i>LOC152225</i>	0.29
<i>PTH1R</i>	17.93	<i>STON1</i>	0.33
<i>CYTH4</i>	15.16	<i>SOX11</i>	0.34
<i>LOC100507584</i>	14.34	<i>RASSF10</i>	0.36
<i>BSND</i>	14.19	<i>KLHL38</i>	0.39
<i>TMIE</i>	10.74	<i>ARL4A</i>	0.40
<i>ENPP2</i>	10.51	<i>ARID5B</i>	0.41
<i>LOXL4</i>	9.85	<i>ABCA1</i>	0.42
<i>IP6K3</i>	8.89	<i>JAG1</i>	0.42
<i>PDK4</i>	8.80	<i>VAV3</i>	0.42
<i>FAM25A</i>	8.65	<i>FAM84A</i>	0.44
<i>HAND1</i>	8.13	<i>SERTAD4</i>	0.44
<i>A4GALT</i>	8.13	<i>PPP1R3C</i>	0.44
<i>WISP2</i>	7.88	<i>RDH10</i>	0.44
<i>VWF</i>	7.67	<i>PPP1R3C</i>	0.62
<i>THRSP</i>	7.65	<i>SMOC1</i>	0.63
<i>CABP7</i>	6.75	<i>KRT17</i>	0.63

Table 3. Most Highly Up-regulated and Down-regulated Genes in the E2 vs E2+ER β Comparison

Up-regulated Genes		Down-regulated Genes	
Gene Symbol	Fold Change	Gene Symbol	Fold Change
<i>ISM1</i>	99.57	<i>ADAMTS5</i>	0.06
<i>FGF19</i>	95.53	<i>STON1</i>	0.15
<i>EGR3</i>	85.29	<i>DKK1</i>	0.16
<i>WISP2</i>	69.21	<i>ADAMTS1</i>	0.16
<i>ESR2</i>	65.01	<i>CHRM1</i>	0.20
<i>THRSP</i>	54.88	<i>HRCT1</i>	0.23
<i>CYTH4</i>	53.59	<i>GRIN2A</i>	0.23
<i>OTOF</i>	51.63	<i>KLHL38</i>	0.24
<i>ENPP2</i>	39.12	<i>NOV</i>	0.24
<i>SCGB3A1</i>	36.32	<i>PPP1R3C</i>	0.25
<i>CD34</i>	29.79	<i>SOX11</i>	0.25
<i>PDLIM4</i>	27.72	<i>IL8</i>	0.26
<i>ANGPTL2</i>	26.90	<i>FGF1</i>	0.26
<i>PGLYRP2</i>	26.83	<i>RASSF10</i>	0.27
<i>LOC727710</i>	25.48	<i>FOXQ1</i>	0.28
<i>SHISA2</i>	25.25	<i>SHROOM2</i>	0.28
<i>CRB2</i>	25.05	<i>ARL4A</i>	0.28
<i>TMIE</i>	24.75	<i>ACER1</i>	0.28
<i>FOXN1</i>	24.04	<i>FAM105A</i>	0.29
<i>MGAT3</i>	23.58	<i>IGFBP3</i>	0.29

ferentiation (Table 4). In addition, genes involved in cell proliferation and cell morphology were enriched in the ER β target genes. Similar molecular and cellular functions were enriched in genes identified in comparisons II and III (Figure 5A), representing potential ligand-independent and ligand-dependent targets, respectively (Supplemental Materials and Methods). The top pathways enriched in the total ER β target gene set included the Wnt/ β -catenin pathway and the G1/S cell cycle checkpoint regulation pathway (Table 5). Indeed, several genes involved in Wnt signaling were verified as ER β target genes, including *DKK1*, *WNT4*, and *CDH1* (Figure 6, B and C). In addition, the gene encoding the cyclin-dependent kinase inhibitor p21, *CDKN1A*, was found to be up-regulated by ER β expression and E2 treatment (Figure 7A). The pathway analysis results support the potential for ER β to regulate key cellular process that may ultimately play a role in growth inhibition.

ER β target genes are regulated in additional breast cancer cell lines and correlate with ER β expression in TNBCs

To confirm that a common set of ER β target genes are shared among TNBC cells, the genes identified in MDA-MB-468-ER β cells were compared to those identified in other TNBC cell lines. First, the gene list was compared to that obtained by Secreto and coworkers (15) in which Hs578T breast cancer cells with inducible ER β expression were used to identify ligand-dependent ER β target

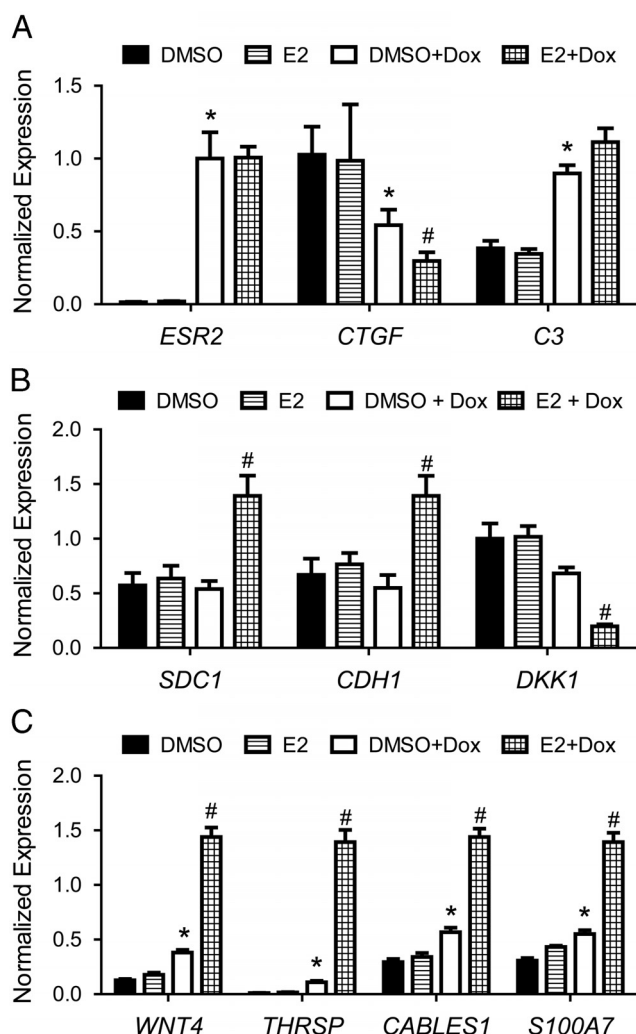


Figure 6. Validation of ER β target genes in 468-ER β #18 cells. 468-ER β #18 cells were treated with vehicle or Dox for 48 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Quantitative PCR was performed as described in the *Materials and Methods*. (A) Expression of *ESR2* and 2 ligand independent target genes: *CTGF* and *C3*. (B) Expression of three ligand-dependent target genes: *SDC1*, *CDH1*, and *DKK1*. (C) Expression of four genes regulated in both ligand-independent and ligand-dependent manners: *WNT4*, *THRSP*, *CABLES1*, and *S100A7*. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control.

genes after 24 hours of E2 treatment. Although the authors used a targeted microarray to assess changes in gene expression, 27 genes were found to be commonly regulated in both MDA-MB-468-ER β and Hs578T-ER β cells (Table 6). Most of these genes were regulated in the same direction, with the exceptions of *BMP5*, *CEBPD*, and *PMAIP1*. Four ER β target genes were then verified in another TNBC cell line, HCC1143, infected with retrovirus encoding green fluorescent protein (GFP) as a control or full-length ER β followed by treatment with DMSO or E2 for 4 hours (Figure 7C). Indeed, all four genes including the cell cycle regulator *CDKN1A* were

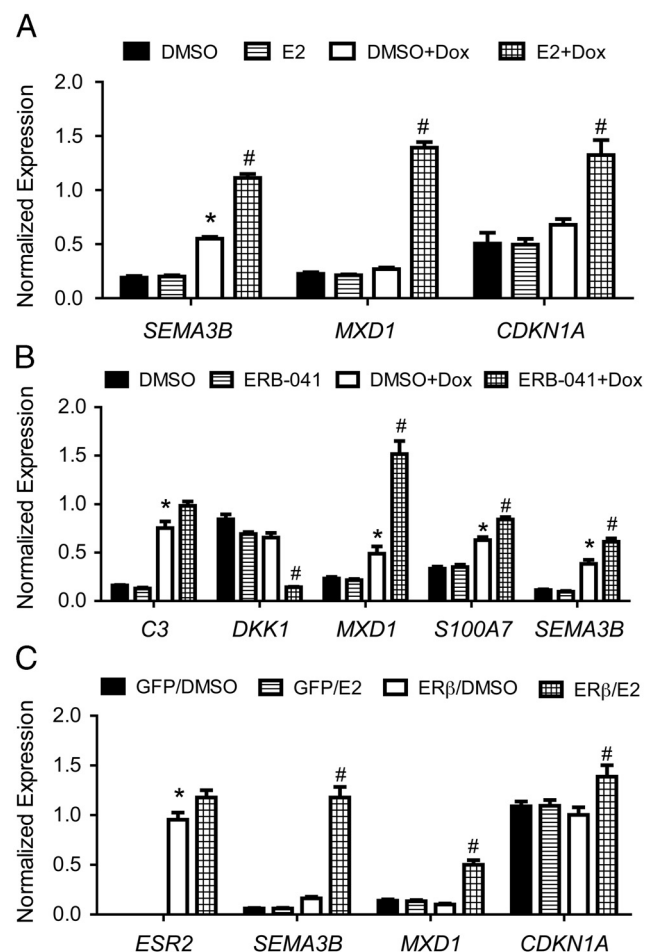


Figure 7. ER β expression regulates common target genes in HCC1143 TNBC cells. (A) Quantitative PCR validation of *SEMA3B*, *MXD1*, and *CDKN1A* in 468-ER β #18 cells treated with vehicle or Dox for 24 hours followed by treatment with DMSO or 10 nM E2 for 4 hours. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control. (B) Quantitative PCR validation of *ESR2*, *SEMA3B*, *MXD1*, and *CDKN1A* in HCC1143 TNBC cells infected with retrovirus for GFP or ER β expression followed by treatment with DMSO or 10 nM E2 for 4 hours. The data represent the mean and SEM of three replicates. *, $P < .05$ compared to GFP/DMSO control; #, $P < .05$ compared to ER β /DMSO control. (C) Quantitative PCR validation of *SEMA3B*, *MXD1*, and *CDKN1A* in 468-ER β #18 cells treated with vehicle or Dox for 24 hours followed by treatment with DMSO or 100 nM ERB-041 for 4 hours. Data represent the mean and SEM of three independent replicates. *, $P < .05$ compared to DMSO control; #, $P < .05$ compared to DMSO+Dox control.

regulated in a similar manner in HCC1143 when ER β was expressed and activated by E2 treatment. These data suggest that ER β regulates multiple common targets across TNBC cell lines.

The ER β target genes identified in MDA-MB-468-ER β cells were next compared to ER β target genes identified by Grober and colleagues (30) in ER α -positive MCF7 cells engineered to express full-length ER β . Using chromatin precipitation followed by deep sequencing for ER β binding sites and gene expression microarrays, they found

Table 4. Top Biological Functions in the E2 vs E2+ER β Gene Set

Top Biological Functions, Molecular and Cellular Functions	P Value	No. of Molecules
Cell movement		
Cell movement	1.21E-18	166
Migration of cells	2.73E-18	154
Cell death and survival		
Necrosis	2.19E-17	201
Cell death	2.66E-16	236
Cellular development		
Differentiation of cells	6.66E-17	129
Proliferation of tumor cell lines	1.54E-12	143
Cellular growth and proliferation		
Proliferation of tumor cell lines	1.54E-12	143
Proliferation of cells	1.70E-12	249
Cellular morphology		
Formation of lamellipodia	4.56E-09	22
Collapse of growth cone	2.31E-07	12

424 “primary” ER β target genes that were differentially expressed when ER β was expressed and contained ER β binding sites within 10 kb of the gene. Of the 930 ER β target genes identified in MDA-MB-468-ER β cells (comparison IV, Figure 5A), 99 genes (10.6%) were also identified as primary ER β targets. Fifty-two of these genes were differentially regulated in cells expressing ER β and not in parental MCF7 cells (Supplemental Materials and Methods). These results indicate that despite the differences in cell lines (ie, ER α -negative basal-like cells versus ER α -positive luminal cells), ER β can regulate common target genes. Most of these genes were also regulated in the absence of ER β or contained ER α binding sites, but three genes were found to contain only ER β binding sites and were only regulated when ER β was expressed: *CCDC103*, *IGSF9*, and *P2RY6*. Although *CCDC103* was only identified in comparison IV (Figure 5A), *IGSF9* was found to be a ligand-dependent target (comparison III) and *P2RY6* was a ligand-dependent target (comparison II) in MDA-MB-468-ER β cells.

Table 5. Top Pathways in the E2 vs E2+ER β Gene Set

Top Canonical Pathways	P Value	Ratio
Hepatic fibrosis/hepatic stellate cell activation	7.24E-07	0.16
Axonal guidance signaling	2.57E-05	0.09
Wnt/ β -catenin signaling	1.91E-04	0.12
Role of IL-17A in psoriasis	3.63E-04	0.39
Aryl hydrocarbon receptor signaling	6.17E-04	0.11
Molecular mechanisms of cancer	8.51E-04	0.09
Glioblastoma multiforme signaling	1.66E-03	0.10
Human embryonic stem cell pluripotency	1.82E-03	0.10
Coagulation system	2.04E-03	0.18
Glutamate receptor signaling	2.45E-03	0.13
Cell cycle: G1/S checkpoint regulation	3.55E-03	0.14

To validate the ER β target genes in TNBCs, a correlation analysis was performed using gene expression data from 43 TNBCs available through TCGA (26). Of the 930 ER β target genes identified in MDA-MB-468-ER β cells (comparison IV, Figure 5A), 109 genes were significantly correlated with *ESR2* expression ($q < .05$, denoted by points in Figure 8; a complete list is provided in Supplemental File 2). Fifteen of these were found to be ligand-independent in MDA-MB-468-ER β (identified comparison II, Figure 5A), and 58 of them were regulated in a ligand-dependent manner (identified in comparison III, Figure 5A). Six of these genes were also identified in Hs578T-ER β cells (highlighted in red in Figure 8), thereby validating that these genes are likely ER β target genes in TNBCs. However, two genes (*ADAMTS1* and *ADAMTS5*) showed a positive correlation with ER β expression but were down-regulated in both cell lines, suggesting that ER β target gene regulation in breast cancer samples is not completely recapitulated by the cell culture models. In addition, two of the genes identified as primary ER β target genes by Grober et al (30) were significantly correlated with *ESR2* expression in the TCGA cohort (highlighted in green in Figure 8), although the correlation coefficients were quite low. This could be due to the difference in breast cancer subtypes: Grober and colleagues used an ER α -positive cell line to identify ER β target genes, while only TNBCs were included in the TCGA analysis. Overall, these results validate that a set of ER β target genes identified in cell culture models are correlated with ER β expression in TNBCs.

Discussion

Because ER β is expressed in 50% to 90% of ER α -negative breast cancers (1–4) and its expression has been associated with a better prognosis in TNBCs (2), ER β may be a therapeutic target for a subset of TNBCs. The results of this study support this hypothesis and confirm the growth inhibitory effects of ER β expression and activation in TNBC cells. However, the requirement of ligand to stimulate ER β 's effects is likely cell context specific. Previously, ER β expression was found to inhibit ER α -negative breast cancer cell growth independent of ligand in MDA-MB-231 and SKBR3 cells (12, 13). In Hs578T TNBC cells, ER β expression inhibited cell growth in a ligand-dependent manner through a G1 cell cycle arrest (15). In culture, ER β expression also induced a G1 arrest in MDA-MB-468 cells, but this effect was both E2-independent and -dependent. One way that ER β may regulate the cell cycle is through up-regulation of the cyclin-dependent kinase inhibitor p21 (encoded by *CDKN1A*), which

Table 6. Common ER β Target Genes Identified in Hs578T-ER β (15) and 468-ER β #18

Gene Symbol	Gene Name	Fold Change MDA468-ER β	Fold Change Hs578T-ER β
<i>ADAMTS1</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 1	0.2	0.4
<i>ADAMTS5</i>	ADAM metalloproteinase with thrombospondin type 1 motif, 5	0.1	0.4
<i>BMP5</i>	Bone morphogenetic protein 5	0.5	2.6
<i>C3</i>	Complement component 3	4.4	12.8
<i>CA12</i>	Carbonic anhydrase XII	10.5	2.9
<i>CD34</i>	CD34 molecule	29.8	2.1
<i>CEBPD</i>	CCAAT/enhancer binding protein (C/EBP), δ	0.5	2.7
<i>CTGF</i>	Connective tissue growth factor	0.3	0.4
<i>DKK1</i>	Dickkopf homolog 1 (<i>Xenopus laevis</i>)	0.2	0.3
<i>EHD2</i>	EH-domain containing 2	2.9	2.3
<i>ENPP2</i>	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	39.1	2.0
<i>FUT8</i>	Fucosyltransferase8 (α [1,6] fucosyltransferase)	2.1	2.1
<i>IGFBP3</i>	Insulin-like growth factor binding protein 3	0.3	0.4
<i>IGFBP5</i>	Insulin-like growth factor binding protein 5	4.6	2.5
<i>JAG1</i>	Jagged 1 (Alagille syndrome)	0.3	0.1
<i>LMO2</i>	LIM domain only2 (rhombotin-like 1)	1.5	3.3
<i>PDE4B</i>	Phosphodiesterase 4B, cAMP-specific	2.0	2.3
<i>PFKFB3</i>	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	4.5	2.1
<i>PIM1</i>	Pim-1 oncogene	2.2	2.3
<i>PLAC1</i>	Placenta-specific 1	2.2	3.1
<i>PLAU</i>	Plasminogen activator, urokinase	0.5	0.4
<i>PMAIP1</i>	Phorbol-12-myristate-13-acetate-induced protein 1	2.1	0.5
<i>SEMA3B</i>	Sema domain, immunoglobulin domain (Ig), (semaphorin) 3B	7.2	3.6
<i>SFRP1</i>	Secreted frizzled-related protein 1	1.8	2.1
<i>TH</i>	Tyrosine hydroxylase	17.7	2.2
<i>TPD52L1</i>	Tumor protein D52-like 1	1.6	2.0
<i>WISP2</i>	WNT1 inducible signaling pathway protein 2	69.2	3.0

regulates the progression from G1 to S phase. Indeed, one of the top pathways identified in ER β target genes was the G1/S checkpoint regulation pathway (Table 5), which includes *CDKN1A*. In MDA-MB-231 TNBC cells, ER β increased the expression of p21 via interaction with another transcription factor, inhibitor of differentiation-1 (14). Future studies are warranted to determine if inhibitor of differentiation-1 plays a role in the regulation of *CDKN1A* in MDA-MB-468 cells.

ER β -mediated growth inhibition may also depend on the cellular environment. ER β was found to elicit growth inhibitory effects in vitro and in vivo in MDA-MB-468-ER β cells. In contrast, in mammary epithelial and breast cancer cells that endogenously coexpress ER α and ER β , ER β -selective ligands were found to induce apoptosis in vitro while promoting tumor growth in vivo (31) by coincubation with a basement membrane extract, suggesting that the cellular environment can modulate ER β activity. Inhibition of extracellular signal-regulated kinase 1 and 2 (ERK1/2) could restore ER β growth inhibitory effects (31). It appears that cellular context modulates ER β growth inhibitory effects. Either the coexpression of ER α or the activation of kinase pathways in the mammary epithelial cell model may account for the differential in vivo effects of ER β in the MDA-MB-468 orthotopic xenograft model.

Like ER α , ER β has two activation functions (AF-1 and AF-2) that confer transcriptional activity. The AF-1 can mediate transcription independent of ligand, and the AF-2 functions in a ligand-dependent manner (32). Although the AF-1 of ER β was found to have negligible activity in a Gal4 assay (33), ER β was found to interact with DNA in a ligand-independent manner (34). In addition, MAP kinase has been shown to phosphorylate the N-terminal activation function (AF-1) of ER β , thereby promoting the recruitment of steroid receptor coactivator 1, which confers ligand-independent transcriptional activity (35). In MDA-MB-468 cells, ER β appears to have both ligand-independent and -dependent activity in regard to target gene expression. Interestingly, most target genes were regulated in response to E2 treatment, suggesting strong ligand-dependent activity in these cells. In contrast, most ER β target genes were regulated independent of ligand in U2OS osteosarcoma cells (36). Therefore, the ligand-independent activity of ER β is likely the result of the availability of cofactors, the activation of signaling pathways, and the promoter context.

Both the ligand-dependent and -independent ER β target genes identified in MDA-468-ER β cells were enriched in genes that regulate cell death, proliferation, movement, and morphology. ER β target genes identified after 24

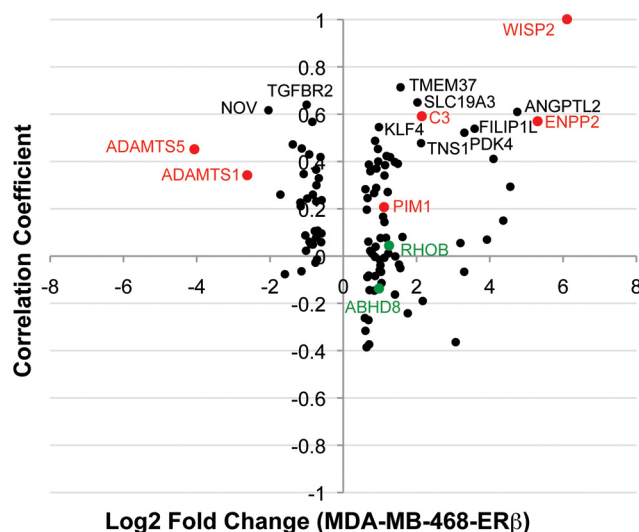


Figure 8. ER β target genes are correlated with ER β expression in a cohort of TNBCs. Gene expression data from the TCGA (26) was used to perform a correlation analysis of ER β target genes and ER β expression. Of the 930 genes identified 468-ER β #18 cells (comparison IV, Figure 5A), 109 genes were significantly correlated with ER β expression ($q < .05$). The correlation coefficient versus the fold change observed in the RNA-seq results for each gene is shown in the scatterplot. The gene symbols of those genes with the highest correlation coefficients are shown on the plot. Genes in red are those that were also identified in Hs578T-ER β cells (15), and genes in green are those that were identified in MCF7-ER β cells (30).

hours of E2 treatment in HEK293 cells were found to be enriched in many of the same pathways (37). A pathway analysis of total ER β target genes revealed significant enrichment of several pathways, including the Wnt/ β -catenin pathway. Of the genes involved in the Wnt/ β -catenin pathway, ER β expression and E2 treatment resulted in the up-regulation of *WNT4* (a noncanonical Wnt ligand) and *CDH1* (a β -catenin interacting protein), as well as the down-regulation of *DKK1* (an inhibitor of Wnt signaling). Down-regulation of *DKK1* by ER β expression and estrogen treatment was also observed in Hs578T breast cancer cells (15) and 293T kidney cells (37). Collectively, the gene expression data suggest that ER β expression may affect the Wnt signaling pathway in breast cancer cells. Although activation of Wnt signaling can transform human mammary epithelial cells (38), a recent study has shown that paracrine Wnt signaling in fibroblasts can either promote or inhibit breast cancer cell growth in mice (39). ER β has been detected in both mammary epithelial and stromal cells (40) and may therefore affect Wnt signaling in a cell type- and cell context-dependent manner.

Given the heterogeneous nature of TNBCs (41), identification of common ER β target genes across all TNBCs would be challenging. Recently TNBC cell lines were classified into basal-like, mesenchymal-like, and luminal-androgen receptor subtypes by correlation with expression

profiles of large breast cancer data sets (42). Although the RNA-seq was performed in basal-like MDA-MB-468 cells, we verified ER β target gene expression in Hs578T, a mesenchymal-like cell line, and HCC1143, another basal-like cell line. Despite differences in the cell line and experimental design, 27 genes identified in MDA-MB-468-ER β cells were found to be ER β target genes in Hs578T-ER β cells treated with E2 for 24 hours (15). Four ER β target genes selected for verification showed the similar regulation by ER β and E2 in HCC1143 and MDA-MB-468 cells (Figure 7C). In addition, an ER β target gene identified in MDA-MB-468-ER β cells, *S100A7*, was found to be an ER β -specific target gene in MCF7 ER α -positive breast cancer cells with inducible expression of ER β and was associated with ER β in ER α -negative breast tumors (43). Several ER β targets were also found to be primary ER β target genes in MCF7 cells (30) and are likely direct targets of the receptor in MDA-MB-468-ER β cells. Most importantly, 109 ER β target genes were correlated with ER β expression in a cohort of 43 TNBCs, including six genes that were also identified in Hs578T TNBC cells. Thus, the genes identified in this study provide the foundation for identifying common target genes of ER β functionality across ER α -negative breast cancers, including TNBCs.

Overall, this study confirms the growth inhibitory effects of ER β in TNBC cells, both in vitro and in vivo, and provides the most comprehensive identification of ER β target genes in TNBC to date. These data also suggest that ER β selective ligands may be useful for targeting ER β in a subset of ER α -negative breast cancers, including TNBCs. ER β -selective ligands could promote the inhibitory effects of ER β while avoiding the proliferative effects mediated by ER α . ER β selective ligands, such as ERB-041, have been found to be well-tolerated (44), supporting the use of these compounds clinically. In addition, the identification of ER β target genes provides a foundation for identifying biomarkers of ER β functionality in TNBCs. Collectively, these data support the hypothesis that ER β may be a therapeutic target in a subset of TNBCs and suggest that ER β may be targeted with ER β -selective ligands that can stimulate its growth inhibitory effects.

Acknowledgments

We gratefully acknowledge Yidan Wang for technical support and Lu Wang for providing the FLuc retrovirus.

Address all correspondence and requests for reprints to: Wei Xu, McArdle Laboratory for Cancer Research, 1400 University

Avenue, University of Wisconsin, Madison, Wisconsin 53705.
E-mail: wxu@oncology.wisc.edu.

This work was supported by the National Institute of Environmental Health and Safety (Grant T32 ES007015), the National Institutes of Health (Grant R01CA125387), the Department of Defense Breast Cancer Research Program (Grants BC100252, Era of Hope Scholar Award W81XWYH-11-1-0237), and the UWCCC (Multi-IT Grant).

Disclosure Summary: The authors have nothing to disclose.

References

1. Skliris GP, Leygue E, Curtis-Snell L, Watson PH, Murphy LC. Expression of oestrogen receptor- β in oestrogen receptor- α negative human breast tumours. *Br J Cancer*. 2006;95(5):616–626.
2. Honma N, Horii R, Iwase T, et al. Clinical importance of estrogen receptor- β evaluation in breast cancer patients treated with adjuvant tamoxifen therapy. *J Clin Oncol*. 2008;26(22):3727–3734.
3. Marotti JD, Collins LC, Hu R, Tamimi RM. Estrogen receptor- β expression in invasive breast cancer in relation to molecular phenotype: results from the Nurses' Health Study. *Mod Pathol*. 2010;23(2):197–204.
4. Yan M, Rayoo M, Takano EA, kConFab Investigators, Fox SB. Nuclear and cytoplasmic expressions of ER β 1 and ER β 2 are predictive of response to therapy and alters prognosis in familial breast cancers. *Breast Cancer Res Treat*. 2011;126(2):395–405.
5. Leung Y-K, Mak P, Hassan S, Ho S-M. Estrogen receptor (ER)- β isoforms: a key to understanding ER- β signaling. *Proc Natl Acad Sci USA*. 2006;103(35):13162–13167.
6. Peng B, Lu B, Leygue E, Murphy L. Putative functional characteristics of human estrogen receptor- β isoforms. *J Mol Endocrinol*. 2003;30(1):13–29.
7. Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. Estrogen receptor β inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. *Cancer Res*. 2004;64(1):423–428.
8. Ström A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor β inhibits 17 β -estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci USA*. 2004;101(6):1566–1571.
9. Murphy LC, Peng B, Lewis A, et al. Inducible upregulation of oestrogen receptor- β 1 affects oestrogen and tamoxifen responsiveness in MCF7 human breast cancer cells. *J Mol Endocrinol*. 2005;34(2):553–566.
10. Hartman J, Lindberg K, Morani A, Inzunza J, Ström A, Gustafsson JA. Estrogen receptor β inhibits angiogenesis and growth of T47D breast cancer xenografts. *Cancer Res*. 2006;66(23):11207–11213.
11. Hodges-Gallagher L, Valentine CD, El Bader S, Kushner PJ. Estrogen receptor β increases the efficacy of antiestrogens by effects on apoptosis and cell cycling in breast cancer cells. *Breast Cancer Res Treat*. 2008;109(2):241–250.
12. Lazennec G, Bresson D, Lucas A, Chauveau C, Vignon F. ER β inhibits proliferation and invasion of breast cancer cells. *Endocrinology*. 2001;142(9):4120–4130.
13. Treeck O, Juhasz-Boess I, Latratch C, Horn F, Goerse R, Ortmann O. Effects of exon-deleted estrogen receptor β transcript variants on growth, apoptosis and gene expression of human breast cancer cell lines. *Breast Cancer Res Treat*. 2008;110(3):507–520.
14. Chen L, Qiu J, Yang C, et al. Identification of a novel estrogen receptor β 1 binding partner, inhibitor of differentiation-1, and role of ER β 1 in human breast cancer cells. *Cancer Lett*. 2009;278(2):210–219.
15. Secreto FJ, Monroe DG, Dutta S, Ingle JN, Spelsberg TC. Estrogen receptor α/β isoforms, but not β cx, modulate unique patterns of gene expression and cell proliferation in Hs578T cells. *J Cell Biochem*. 2007;101(5):1125–1147.
16. Hartman J, Ström A, Gustafsson JA. Estrogen receptor β in breast cancer—diagnostic and therapeutic implications. *Steroids*. 2009;74(8):635–641.
17. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS. Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) α or ER β in human osteosarcoma cells: distinct and common target genes for these receptors. *Endocrinology*. 2004;145(7):3473–3486.
18. Shanle EK, Hawse JR, Xu W. Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. *Biochem Pharmacol*. 2011;82(12):1940–1949.
19. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature*. 2005;436(7050):518–524.
20. Li B, Ruotti V, Stewart RM, Thomson JA, Dewey CN. RNA-Seq gene expression estimation with read mapping uncertainty. *Bioinformatics*. 2010;26(4):493–500.
21. Faulkner GJ, Forrest AR, Chalk AM, et al. A rescue strategy for multimapping short sequence tags refines surveys of transcriptional activity by CAGE. *Genomics*. 2008;91(3):281–288.
22. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods*. 2008;5(7):621–628.
23. Taub MLD, Speed TP. Methods for allocating ambiguous short-reads. *Comm Inform Syst*. 2010;10(2):69–82.
24. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;12:323.
25. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol*. 2010;11(10):R106.
26. Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. *Nature*. 2012;490(7418):61–70.
27. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc B (Method)*. 1995;57:289–300.
28. Storey JD, Tibshirani R. Statistical significance for genomewide studies. *Proc Natl Acad Sci USA*. 2003;100(16):9440–9445.
29. Harris HA, Albert LM, Leathurby Y, et al. Evaluation of an estrogen receptor- β agonist in animal models of human disease. *Endocrinology*. 2003;144(10):4241–4249.
30. Grober OM, Mutarelli M, Giurato G, et al. Global analysis of estrogen receptor β binding to breast cancer cell genome reveals an extensive interplay with estrogen receptor α for target gene regulation. *BMC Genomics*. 2011;12:36.
31. Cotrim CZ, Fabris V, Doria ML, et al. Estrogen receptor β growth-inhibitory effects are repressed through activation of MAPK and PI3K signalling in mammary epithelial and breast cancer cells. *Oncogene*. 2012;32(19):2390–2402.
32. Heldring N, Pike A, Andersson S, et al. Estrogen receptors: how do they signal and what are their targets. *Physiol Rev*. 2007;87(3):905–931.
33. Cowley SM, Parker MG. A comparison of transcriptional activation by ER α and ER β . *J Steroid Biochem Mol Biol*. 1999;69(1–6):165–175.
34. Hall JM, McDonnell DP. The estrogen receptor β -isoform (ER β) of the human estrogen receptor modulates ER α transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology*. 1999;140:5566–5578.
35. Tremblay A, Tremblay GB, Labrie F, Giguère V. Ligand-independent recruitment of SRC-1 to estrogen receptor β through phosphorylation of activation function AF-1. *Molecular Cell*. 1999;3(4):513–519.
36. Vivar OI, Zhao X, Saunier EF, et al. Estrogen receptor β binds to and regulates three distinct classes of target genes. *J Biol Chem*. 2005;280(29):22059–22066.
37. Zhao C, Putnik M, Gustafsson JA, Dahlman-Wright K. Microarray

- analysis of altered gene expression in ER β -overexpressing HEK293 cells. *Endocrine*. 2009;36(2):224–232.
38. Ayyanan A, Civenni G, Ciarloni L, et al. Increased Wnt signaling triggers oncogenic conversion of human breast epithelial cells by a Notch-dependent mechanism. *Proc Natl Acad Sci USA*. 2006;103(10):3799–3804.
39. Green JL, La J, Yum KW, et al. Paracrine Wnt signaling both promotes and inhibits human breast tumor growth. *Proc Natl Acad Sci USA*. 2013;110(17):6991–6996.
40. Palmieri C, Saji S, Sakaguchi H, et al. The expression of oestrogen receptor (ER)- β and its variants, but not ER α , in adult human mammary fibroblasts. *J Mol Endocrinol*. 2004;33(1):35–50.
41. Shah SP, Roth A, Goya R, et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature*. 2012;486(7403):395–399.
42. Lehmann BD, Bauer JA, Chen X, et al. Identification of human triple-negative breast cancer subtypes and preclinical models for selection of targeted therapies. *J Clin Invest*. 2011;121(7):2750–2767.
43. Skliris GP, Lewis A, Emberley E, et al. Estrogen receptor- β regulates psoriasis (S100A7) in human breast cancer. *Breast Cancer Res Treat*. 2007;104(1):75–85.
44. Roman-Blas JA, Castañeda S, Cutolo M, Herrero-Beaumont G. Efficacy and safety of a selective estrogen receptor β agonist, ERB-041, in patients with rheumatoid arthritis: a 12-week, randomized, placebo-controlled, phase II study. *Arthritis Care Res (Hoboken)*. 2010;62(11):1588–1593.



Join The Endocrine Society and network with
endocrine thought leaders from around the world.

www.endo-society.org/join